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THE UNIVERSITY OF ALBERTA

MICROWAVE THAWING OF MAMMALIAN CELLS, TISSUES, AND ORGANS.

by

© RAY V. RAJOTTE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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EDMONTON, ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty Studies and Research, for acceptance, a thesis entitled Microwave Thawing of Mammalian Cells, Tissues, and Organs submitted by Raymond Victor Rajotte in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

A system of electrically controlled freezing with fluorocarbon (FC47) and thawing with microwave at 2450 MHz is described, and this system tested using frozen whole canine kidneys in order to study uniformity of heating. It was found that heating uniformity is very dependent on proper perfusion of the kidney with dimethylsulphoxide (DMSO). Various other techniques such as tissue histology, reimplantation in the dog, and microfil injections for microcirculation study were also used to assess different aspects of whole kidney viability.

The principal conclusion reached from the whole organ studies is that, for uniform microwave thawing, adequate perfusion is essential at the three critical stages: initial rinsing, addition of protective agents and cooling with fluorocarbon.

In the one kidney reimplanted following the freeze-thaw insult, it was found that after five days some vascular integrity was maintained, even though the functional capacity of the kidney did not return.

Slices of kidney were used in evaluating different cryophylactic agents, and different freezing rates. Waterbath and microwave thawing were compared, at varying thawing rates. Viability of slices was assessed using the Warburg appartus to

determine oxygen consumption and uptake of para-aminohippurate. Histological studies at various stages show the difficulties inherent in drawing conclusions about viability from such studies alone.

Different cryophylactic agents were also evaluated at a cellular level. In these studies, viability of cell cultures was used to compare the effects of microwave and waterbath warming and establish the value of microwave in thawing. Throughout the investigations, relatively rapid thawing rates (up to 5°C/second) were employed.

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CHAPTER I

INTRODUCTION

"The Problem of Anabiosis, or Latent Life, History and Current Concept" was the title of the 9th Leeuwenhoek lecture delivered by Keilin in 1958 (1). This classic work reviewed all the significant studies on the natural processes of anabiosis, now referred to as cryptobiosis, and the laboratory attempts at inducing latent life, from the observations of Henry Power, M.D., in 1661 (2a), and Antony van Leeuwenhock in 1702 (2b), through the intensive investigation period of the 1930's (3), when dehydration and vitrification effects were studied in detail, to the birth of modern cryobiology, with the work of Parkes (4), Smith (5), Lovelock (6) and others, at the end of the 1940's. Detailed reports on the preservation of cells, spermatozoa, cornea, etc., have been given in the Journal of Cryobiology (1964). In 1966 the state of the art of cryobiology was recorded in a book by Meryman (7). Subsequent books (8), (9), have described work at the cellular level as well as current clinical techniques, up to 1970. A recent article by Mazur (10) reviewed freeze-protective mechanisms, in particular the effects of different cryoprotective agents. In 1972 and 1973, a number of papers were published relating to survival of 2 - 8 cell embryos from the deep frozen state (11), to pacemaker activity with thawed myocardial

cells (12) and to attempts to preserve kidneys (13) and hearts (14). As was stated by Meryman (7) in 1966, and verified by Pegg (44) in 1972, little progress has so far been made in the viable preservation of large organs, particularily the kidney.

As transplantation surgery and immunology advance, the need for longer periods of organ storage is increasing. Storage in the past has been attempted in various ways, but the most common methods used are: normothermic perfusion, hypothermic storage, hypothermic perfusion, hyperbaric oxygen and hypothermic storage, and storage by freezing.

Starling and Verney (15) in 1924 maintained ex vivo dog kidneys normothermically by whole blood perfusion, but even with the use of the modern heart-lung machine, preservation has not exceeded seven hours, which makes the method impractical.

Hypothermic storage has extended the viable preservation time to twelve hours. The metabolic activity of kidneys stored just above freezing is reduced to 1/5 of normal. Storage for twelve hours is possible at 0 to 5°C, with storage time dropping to seven hours at temperatures of 5 to 10°C, and to two hours at temperatures of 15 to 25°C. Using the above techniques, hearts and lungs can be stored for a considerably shorter time (16), (17), (18).

Hypothermic perfusion has proved very successful in attempts to increase storage time. Canine kidney perfused with pre-cooled blood from the same animal followed by storage at 0°C without perfusion has allowed viable preservation for up to twenty-four hours (19). Humphries (20), (21), (22) has shown that

dog kidneys can be stored for twenty-four hours at 10°C when perfused at 30 - 40 mmHg. with oxygenated plasma or serum. Forty-eight hour storage was obtained when dilute blood was passed through a glass wool filter during 10°C perfusion of canine kidney. Four to five day storage has been obtained when culture media was used as the perfusate for similar organs at 10°C (23), (24). Pegg and Farrant (25) have shown that many problems occured when a balanced salt solution at a physiological flow rate was used. Belzer (19) has perfused dog kidneys at 50 to 80 mmHg. using cryoprecipatated oxygenated plasma at 10°C as the perfusate. Up to seventy-two hour storage has been achieved with human kidneys using the above technique. Proctor and Parker (26) have stored canine hearts for seventy-two hours using a similar procedure. This technique has not been as successful on the liver (27), (28) though Brettschneider *et al* (29) has maintained dog liver viability for twenty-four hours using hypothermic perfusion.

In the hope of increasing preservation time, Lillehei has employed storage at 0°C with hyperbaric oxygen. This group obtained seventy-two hour storage at a pressure of fifteen atmospheres (30), (31), (32), (33), (34). In subsequent work, reliable results have been obtained for kidney storage for twenty-four hours using hyperbaric oxygen at 4.4 atmospheres (35).

From the literature, it would seem that the greatest success in organ storage above 0°C is by the use of hypothermic perfusion.

The clinical need for organ storage banks has been clearly stated by Dausset (36). From a consideration of the twenty-

six principal HL-A transplantation antigens, it would be necessary to have a waiting list of several hundred patients in order to obtain a 95% certainty of having a suitable recipient for a donor kidney. In practice, an isolated kidney will maintain acceptable viability for only about seventy-two hours in the unfrozen state at +4°C, thus imposing immense constraints on the transplantation of donor and cadaver kidneys. Similar constraints apply to heart transplantation (37).

The ultimate goal in organ preservation would be to lower the metabolic rate of the organ to almost zero, which can only be done by lowering the temperature of the organ to absolute zero, as yet unattainable. It is generally assumed that organs can be stored indefinitely in the frozen state, once the frozen state is successfully achieved. However, there is some indication of increased metabolic activity below -200°C (38), which suggests the existence of an optimum storage temperature. This will have to be investigated before long term storage is feasible. It is now possible to store sperm for ten years with good viability upon thawing at a temperature of -196°C (39), but otherwise this aspect of organ preservation is rather sparsely covered in the literature, and it is certainly a possible area for future research.

When the temperature of an organ is lowered past the eutectic point, various phenomena begin to occur. With slow freezing there is an increase in concentration, expecially of extracellular electrolyte, and water leaves the cell. Fast freezing causes intra-cellular ice crystals, as there is insufficient

time for the water to pass out of the cell in response to the hyper-osmotic gradient established by extracellular ice formation. To counteract these two effects, cryophylactic agents are used. It is believed that the presence of the protective agents lowers the eutectic point so that the damaging effects become tolerable. There are two main types of cryophylactic agents: a) those which permeate the water space of the whole tissue, such as dimethylsulphoxide (DMSO) and glycerol, and b) those which are extracellular and lessen the rise in extracellular electrolyte concentration as ice forms, as well as stabilising the cell membrane. Such agents are mannitol, sucrose and to some extent, glucose. Cell suspensions, sperm, red blood cells, and some non-vascularised tissues such as cornea have been stored in subzero storage banks with great success (40). The same success has not been enjoyed in whole organ subzero preservation. In a multi-cellular system, such as the kidney, the problems of uniformity of freezing, freezing rates, protective agents, and methods of thawing become very apparent. In 1965 the 3M Company developed the compound Fluorocarbon which at that time was used as an insulator in transformers. This compound had the properties of being non-toxic to biological material, and the capabilities of carrying O_2 and CO_2 equivalent to hemoglobin, but in linear fashion. These properties stimulated interest in the organ preservation research area (41), (42), (43). Of greatest interest in the cryobiology field however, was that it remains liquid until -30 or -40°C, a property that is very useful for uniform organ perfusion below zero.

For the past two decades a considerable amount of work has been done in trying to find the best protective agent for whole organ preservation. In a multicellular system, what may be optimum for one type of cells, may not protect a second type in the same organ. In a recent paper, Pegg (44), pointed out that glycerol was superior to DMSO when used as a protective agent in kidney freezing. He showed that DMSO caused damage to the endothelial lining of the blood vessels, a structure that must be preserved if whole kidney freezing is to become a reality.

In the quest for successful whole organ preservation by freezing, investigation of possible methods for thawing has lagged behind the study of freezing techniques. Microwave rewarming of organs was first reported by Lovelock in 1953 (45) and subsequent investigations have been made by Kenney *et al* (46), Holst *et al* (47), Hamilton *et al* (48), and Ketter *et al* (49), (50). The difficulties encountered by Lehr *et al* (51), even with the heating uniformity possible with microwaves, are very apparent.

A problem following the freeze-thaw insult is the method of assessing the damage incurred, and deciding if the frozen-thawed organ is still viable. The best test for viability is reimplantation, as there is no in vitro method of evaluation which can completely replace this step, evaluating any organ storage system requires tests that are more convenient and less committal. Abbot (52) defines viability as possessing the qualities of life, but goes on to say that the problem is in understanding the qualities of life. Cells have the functions of

motility, metabolism, respiration, reproduction and phagocytosis, but as Abbot (52) emphasizes, these qualities are only manifestations of cell vitality and do not really describe viability itself.

Viability of an organ is thus difficult to measure, but we can assess characteristics at the three basic levels of organization: cellular, tissue slice, and whole organ. Assessment at each level gives some idea of viability. Assays at the cellular level may be an indication of cell viability but it is difficult to translate these to the whole organ level. Associated with the frustrations of viability assay in studies on freeze-thaw survival, Sherman (53) states: "It is the pessimist's assessment of life. Life, life, what is life? We are born and we die. What happens in between only aggravates us. As optimists, we should happily pursue our interest in viability assay, expecting continuous enlightenment, along with aggravations, of course."

The following chapters describe and illustrate techniques used in assessing viability after freezing and thawing at the cellular, tissue, and organ level. An essential prerequisite to any viability study is uniformity and control in the freeze-thaw cycle. Equipment which approaches satisfaction of these criteria was developed for each of the three levels, with only small modifications in each case.

CHAPTER II

METHODS

A. FREEZING AND THAWING OF KIDNEY SLICES AND WHOLE KIDNEYS

The following sections describe the method used in freezing whole kidneys uniformly by use of FC47 perfusion. To rewarm the kidney, a microwave system was built to attempt to obtain fast uniform heating. Following the freeze-thaw insult, routine histology sections, microfil injection preparations and reimplantation were all used to study different aspects of preservation. Kidney slices were also studied to see the effect of freezing and microwave heating. The slices were analyzed in the Warburg apparatus (56) for O_2 and para-aminohippurate $+I^{131}$ (PAH#) uptake followed by hematoxylin and eosin (H&E) histological studies.

In acute experiments whole kidneys and kidney slices were frozen, stored in dry ice or liquid nitrogen, thawed and studied according to methods described in the following sections. In a small number of experiments, kidneys were reimplanted after various manipulations and studied chronically. The kidneys were obtained from healthy mongrel dogs.

1. FREEZING

(a) Whole kidneys. A vasodilator (dibenzylene) was injected into the dog prior to nephrectomy. After removal, each kidney was rinsed

for five minutes with lactated Ringer's (Baxter) at 25°C. When whole kidneys were to be frozen, dimethylsulfoxide (DMSO) was added to Ringer's in a two step perfusion process. 10% (v/v) DMSO in Ringer's was perfused for ten minutes followed by 20% (v/v) DMSO in Ringer's for a further ten minutes. During this period of DMSO uptake, at either 4 or 25°C, the kidney was immersed in a bath of lactated Ringer's at 4°C.

The kidney was then transferred to the temperature controlled perfusion freezer showen in Figure 1.

Figure 1. *Freezing chamber.* A liquid nitrogen jet impinges on the cooling coil (A) which carries the perfusate (fluorocarbon) into the organ's artery (D). The perfusate is collected in the tray (F) from the vein cannula (E) and returned through the bubble-trap (G) to the cooling coil. The liquid nitrogen valve in the lower chamber is controlled by the thermocouples (B, C, & H). To pressure transducer (I).

Cooling was achieved by continuous perfusion with fluorocarbon (FC47, 3M Co.) cooled by a liquid nitrogen jet. As DMSO is not soluble in FC47 it would not be removed from the tissue by fluorocarbon perfusion. The controlled cooling system is shown diagrammatically in Figure 2. *

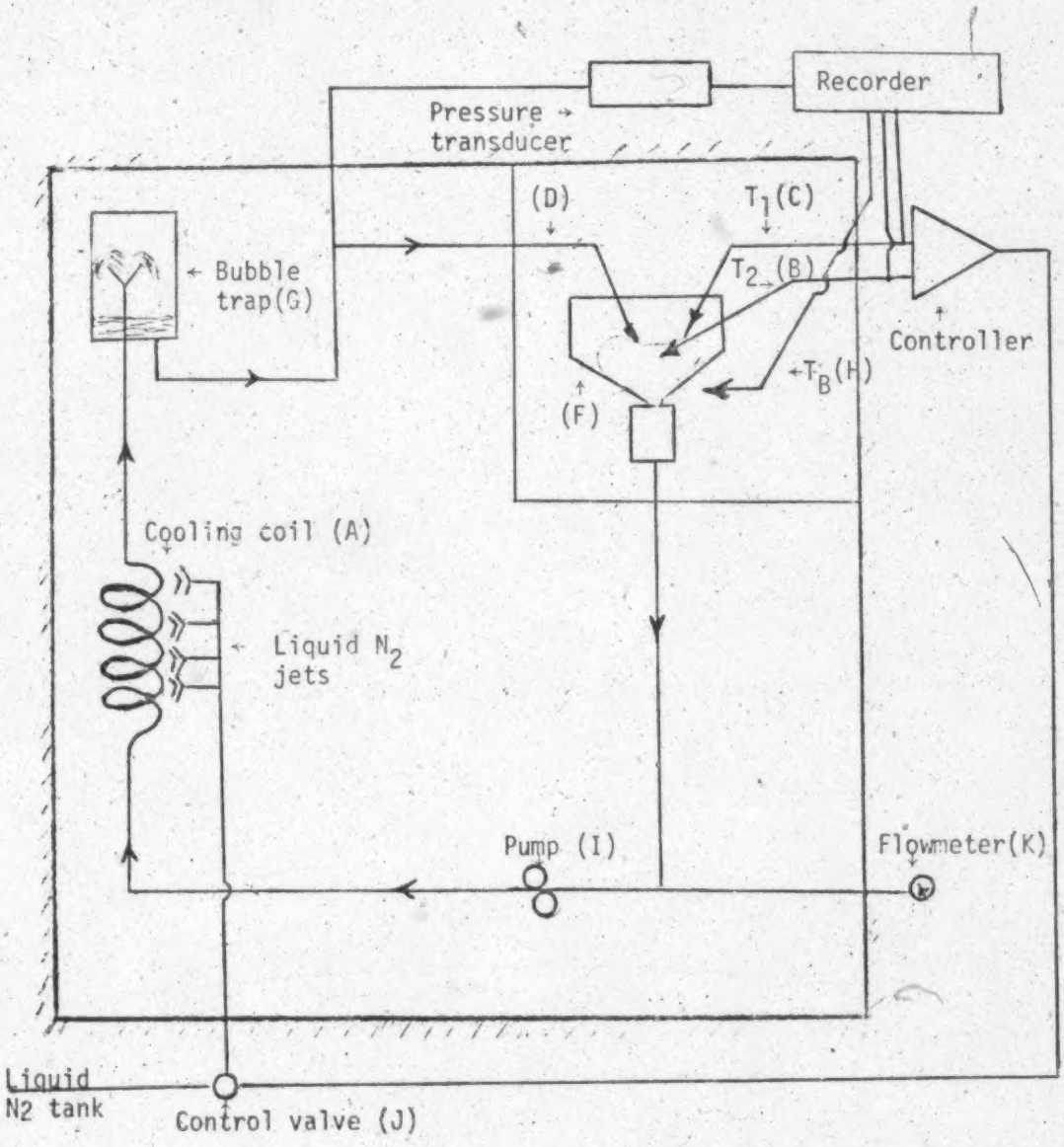


Figure 2. *Line diagram of organ cooling system.*

* Appendix 1 Circuit diagrams for controller.

Thermocouples placed in the cortex (T_1), ureter (T_2) and environment of the chamber (T_B) were connected to a differential amplifier which operated the control valve on the liquid nitrogen jets. Cooling only proceeded if the temperature differential across the organ, $T = [T_1 - T_2]$ ($T_B \approx T_2$) was less than a present value; we chose $T \leq 5°C$ and achieved an average cooling rate of approximately 1°C/minute from +4 to -40°C. -30°C to -40°C is the lowest temperature at which fluorocarbon would perfuse (10cc/minute at 200 mm Hg). No pressure was maintained on the venous side, the affluent draining by gravity into the reservoir. The flow rate at 4°C was 80 - 100 cc/minute when the pressure was 100 mm Hg. (The flow rate was adjusted during cooling to ensure that the pressure, monitored just proximal to the needle tied into the artery, never exceeded 200 mm Hg.). When the flow had effectively stopped, at about -30 to -40°C, external cooling was used, with ΔT maintained at <5°C. The frozen kidney at a temperature of -79°C was stored in dry ice for periods not less than three hours and in some cases several days. The thermocouples were carefully removed before storage.

(b) Kidney slices. Thin (0.028" thick) sections, weighing between 0.1 and 0.3 g were cut from kidneys which had been rinsed immediately after removal from a dog with lactated Ringer's solution. The slices were placed in Cross solution (56) or a culture medium (specified in the Results Tables for each case) with 10 or 15% DMSO and allowed to incubate for twenty minutes, with mechanical agitation and by bubble oxygenation. In some cases,

the slices were then transferred to an emulsion of Ringer's and fluorocarbon, the emulsifying agent being pluronic F68 (polyethylene oxide glycol, Wyandotte), itself a high molecular weight cryo-protective agent (54), (55).

From each batch of twenty slices, five were taken as controls and placed in a Warburg apparatus (56). The remainder were cooled down to -100°C in 10 ml glass vials containing various solution, A - G, at rates between 0.5 and 0.7°C/minute before being transferred directly to liquid nitrogen storage. After thawing in a waterbath or by microwave heating, these samples were transferred to the Warburg apparatus where O_2 and I^{131} labelled para-aminohippurate (PAH#) uptake measurements were made.

2. THAWING

(a) Whole kidneys. Frozen organs were placed individually in a teflon holder and gently clamped into position by teflon bolts, Figure 3. The supported organ was then immersed into a glass container filled with fluorocarbon at 4°C. The assembly was then placed on a teflon table in the microwave heating system, a resonant multimode cavity shown in Figure 4.* After thawing, differential kidneys were perfused with Ringer's lactate containing either 10%DMSO or 10% Mannitol, initially, and then Ringer's lactate. This perfusion step was calculated to displace the hyperosmolar fluorocarbon from the vascular tree and remove DMSO from the hyperosmolar interstitial and intracellular fluid by diffusion without subjecting the cells to an excessive osmotic gradient.

* Appendix 2 Discussion on design of multi mode cavity.

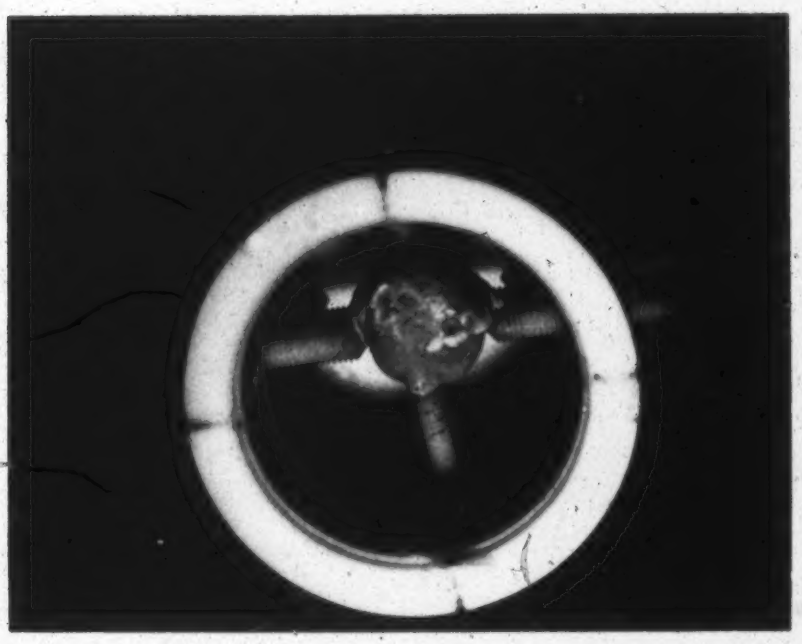


Figure 3. *Teflon holder.* Frozen canine kidney clamped lightly in teflon holder. The holder fits into a beaker of fluorocarbon which is inserted into the microwave oven.

At the time the connections were made to do this, internal temperatures were recorded by a thermocouple needle. Some experiments were terminated at this point and the thawed organ studied for temperature gradients and the effectiveness of the initial perfusion. In experiments where DMSO was removed, Ringer's lactate containing 10% DMSO was first perfused for 10 minutes, followed by a 10 minute perfusion with Ringer's alone. Mannitol, in decreasing concentration, was also used to remove the DMSO while at the same time minimising the osmotic gradients across cell walls.

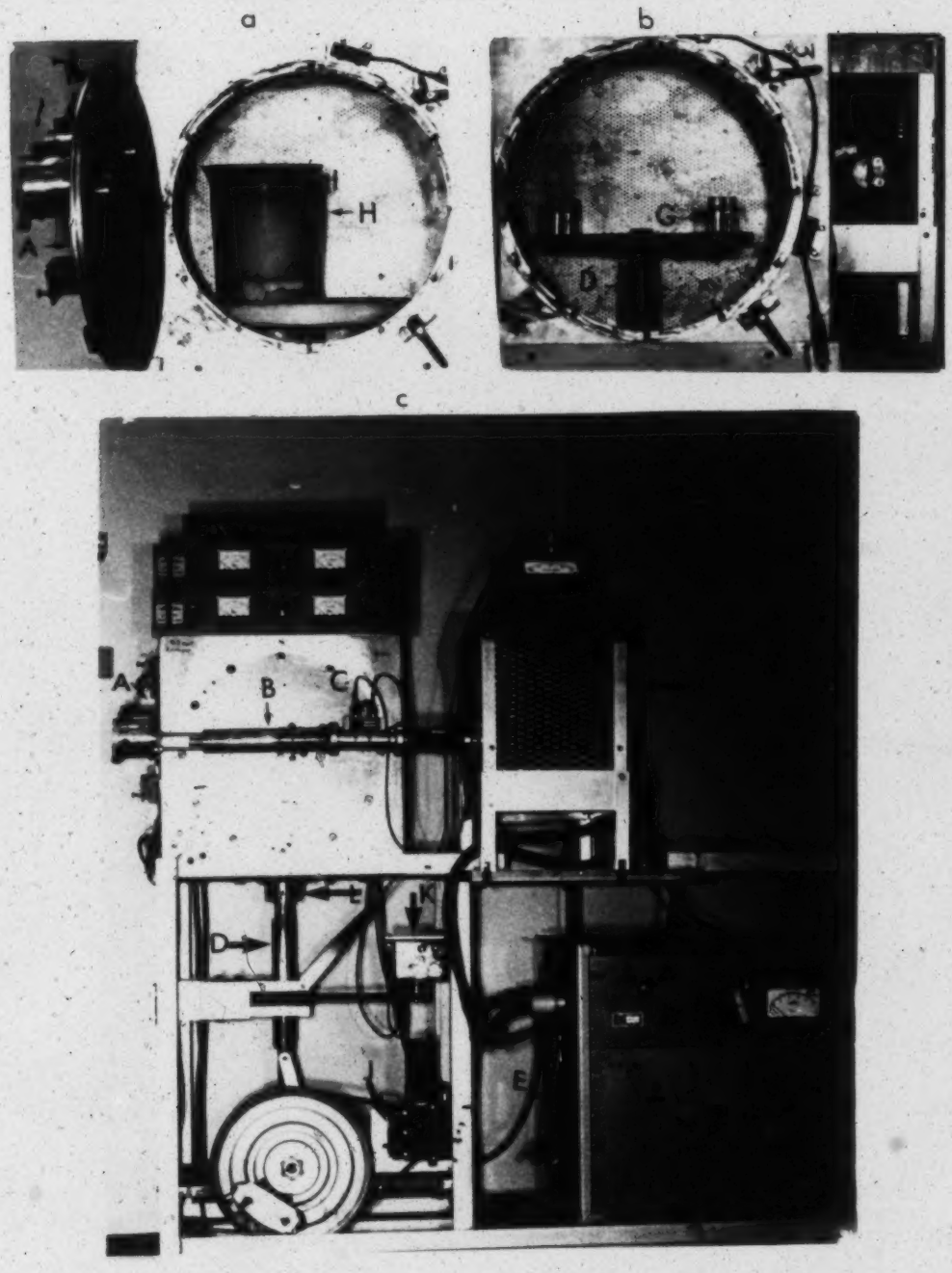


Figure 4. *Microwave thawing system: 1.6 kw at 2450 MHz.* A: cavity door, with interlocks and tuning posts. B: side wall with antenna and power coupling unit. C: power monitor on transmission line. D: mechanical for oscilating turntable. E: magnetron power supply and control system (Amperex Corp.). F: 2 kw (Philips) 2450 MHz magnetron encased with protection systems. G: (inset c) turntable rack for slice holders. H: (inset b) teflon holder in polyethylene beaker. I: mesh facing on inside cavity wall to increase wall loss and enhance coupling. J,K: variable speed dc driving shaft through wave trap L. independently adjustable in speed.

The transfer time from the liquid nitrogen to the microwave system was typically two minutes; the thawing time (see results) was of the order of one minute. The total time that a kidney had been maintained without normal circulation above +4°C was about one hour before subsequent manipulations (for microfil injection, histology or reimplantation).

(b) Slices. The frozen slices were thawed by placing the glass vials directly into the microwave system (Figure 4b). By reducing the input power level and loading the oven with water samples, a technique used in the study of microwave thawing of tissue culture cells described in the following chapter, the rates of controlled heating ranged between 0.1 and 10°C/second. The more uniform heating rates achieved with the whole kidneys (2 - 4°C/second) were also used with the slices.

The microwave cavity has dimensions 48.5 X 40.0 X 38.5 cm dimensions which give rise to a large and fairly even number of modes of resonance over the bandwidth of the magnetron, when the cavity is lightly loaded (57). The stripline antenna, coupling the transmission line B to the cavity in Figure 4, is not shown in the Figure but is one of several antennae studied for the work. The design of the antenna used is due to Johnston (58), and gave satisfactory results for a wide range of frozen loads in the cavity, provided these loads are moved through the electromagnetic fields in the cavity in a rapid and random fashion. Figure 4 shows the method used: the rotating turntable, driven by motor K,

is oscillated by motor J through a flywheel and lever. Although the method is rather crude, and an arbitrary predetermined set of path lengths and velocities were used, the method has proved surprisingly effective. (A different method, which might be advantageous if the technique were ever to become a clinical reality, is described in the Discussion section). In the cavity system, the fluorocarbon and teflon absorb a negligible amount of microwave power. The function of this large mass, we postulate, is to enclose the organ in a quasi-resonant liquid chamber coupled to the microwave oven, the coupling resonant field patterns of which are continuously stirred (or changed) by the movement. On a time average basis, 50% of the incident power is reflected back to the magnetron, leaving in our system a maximum of 800 watts coupled to the cavity. Of this, about 75% is transferred to the organ and absorbed power density (at the maximum setting) is of the order of 10 watts/mg. The remainder of the power is absorbed in the cavity walls. The actual thawing rate can be changed, for coupling or the loading of the cavity, the latter being possible by adding stationary water samples.

Perfect uniformity of heating with whole kidneys has not, however, been achieved as yet, but the variations are probably acceptable, i.e. a final temperature of 20±10°C. In determining heating times empirically, we assume that if all the tissue is above the freezing point and below 36°C, we are within a viable range. Hence, an average temperature of 20°C allows variations up to ±15°C with a factor of safety.

3. UNFROZEN KIDNEYS

To study the microwave insult and the effects of the cryoprotective agent, some kidneys were not frozen; they were perfused and cooled to +4°C by the method described above and immediately rewarmed in the microwave system. Kidney slices were also studied, warming them from 4°C to 35°C, followed by O_2 and $PAH^{\#}$ uptake in the Warburg.

4. MICROFIL

This injection technique was used to outline the microcirculation of the kidney. Microfil (59) is a catylisal silastic rubber, which will pass through the glomeruli and post-glomerular capillary circulation into the venous system. The kidney was sliced, after the microfil had set, the tissue was dehydrated with increasing concentrations of alcohol and then made transparent with methyl salicylate. Photographic records were taken at a magnification of 40 to 65 times.

5. HISTOLOGY

Hematoxylin and eosin preparations were prepared for the sample by the usual routine staining procedures, in some cases after microfil injection of whole kidneys. In assessing these stained preparations a 1 to 4 grading system was used: 1 being normal and 4 being complete loss of normal structure. The glomeruli, tubules, vessels and interstitium were graded separately. Arbitrary criteria of damage had to be devised as the tissue had been subjected to various insults under circumstances which precluded

the normal inflammatory response to tissue injury, responses which are normally mediated by biochemical substances and cellular elements in blood. The arbitrary criteria included swelling of cells, disruption of glomerular capillary wall or the walls of renal tubules, loss of glomerular or tubule cell nuclei, separation of tubules to give wide interstitial spaces, loss of appearance of tubules cell cytoplasm, roughening of arterial and arteriolar lining endothelium, and disruption of the elastic membrane in arterial and arteriolar walls. Finally, each slide was assigned a general overall grading, v - "viable", pv - "possibly viable", pnv - "probably not viable", nv - "not viable", dnv - "definitely not viable". Although the word "viable" is used in this general assessment its use is also quite arbitrary as it could not be correlated with experience with reimplantation. However, these criteria were deemed to be the best that could be devised for assessment of tissue damage in our experiments to date.

6. REIMPLANTATION

One kidney was reimplanted for five days and then removed for histologic and microfil examination. Other kidneys were reconnected externally to the femoral artery and vein of the same dog, and allowed to perfuse for three to four hours. Assessment of this tissue, after blood recirculation, was in accord with usual pathologic criteria. As might be expected, after recirculation, evidence of tissue damage was much greater than could be detected in thawed tissue prior to re-perfusion with normal blood.

7. TEMPERATURE PROFILES

Some kidneys, at different stages, were used to study temperature profiles. For all temperature measurements, 25 gauge copper-constantan thermocouples were used with a Leeds Northrop potentiometer equipped with a standard cell (Model 8693-2). Profiles, as opposed to isolated temperature measurements within an organ, were taken by cutting a kidney in the medial plane and probing beneath the surface of the different area.

8. O_2 AND PAH# UPTAKE TESTS (WARBURG APPARATUS)

Two biochemical parameters were used to assess viability: oxygen uptake in a Warburg apparatus (with carbon dioxide absorption) and the uptake of I^{131} para-aminohippurate as judged by slice/medium (s/m) concentration ratios (52), (56) at the end of a period of slice incubation. Respiration is a crude index for intact cell metabolism as isolated mitochondria can respire (60), I^{131} PAH# uptake, a function of proximal tubule cells, is dependent on complex mechanisms, including the membrane sodium and potassium dependent ATP-ase, and is much more sensitive an index of intact cell viability.

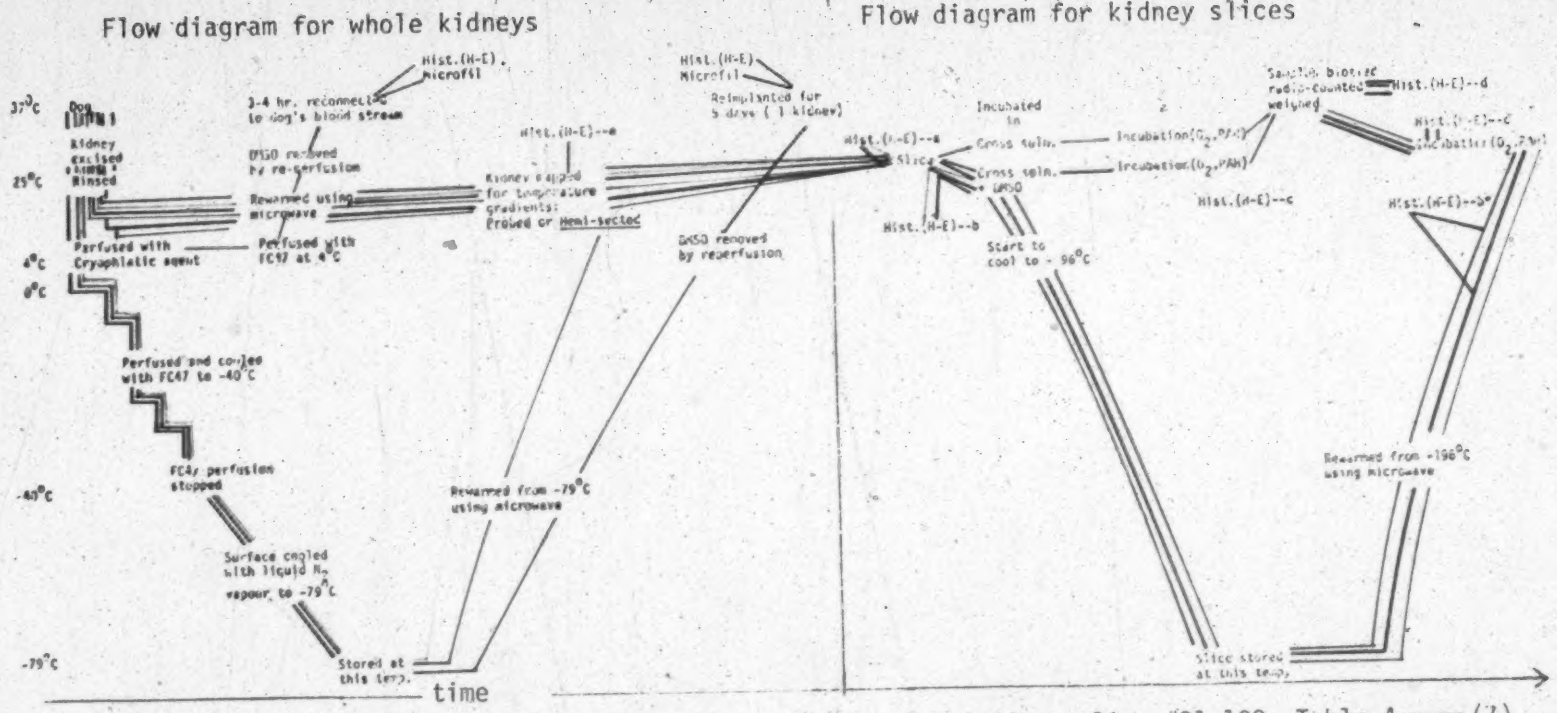


Figure 5. Kidney #8,9,10,11,16, Table 1——(1)
Kidney #13, 14, 15, 17, 18, Table 1——(2)
Kidney #1,2,3,4,5,6,7, Table 1 (3)
Kidney implanted——(4)
Kidney slice #75, 76, 77, 31, 32, 39, 40, 41, Table 2——(5)
Kidney slice #36, 37,38, Table 2——(6)
Kidney slice #81-102, Table 4——(7)
Kidney slice #102-113, Table 5——(8)
Kidney slice #114-117, Table 5 (9)
Kidney slice #118-122, Table 5——(10)

CHAPTER III

EXPERIMENTAL RESULTS ON WHOLE KIDNEY AND KIDNEY SLICES

Freezing and microwave thawing of whole kidneys. Experiments on the relationship of adequacy of perfusion of whole kidney to the uniformity of organ temperatures after microwave rewarming was researched by using the perfusion system, described in the previous chapter: perfusion with a cryophylactic agent (such as DMSO) and then cooling to -40°C with FC47 perfusion, surface freezing to -79°C, microwave rewarming and then measurement of temperature gradients by probing intact kidneys or after hemisection, and comparing these with routine histology and the preservation of the microcirculation after microfil injections.

RESULTS

The data from the experiments are presented as follows:

(a) The three sets of experimental procedure are summarized and coded for color in Figure 6 to assist the reader.

(b) Certain details for each color-coded sub-group are listed in Table 1.

(c) Using Table 1, to obtain the appropriate page number, data of microwave procedure, histology, microfil injection, and tissue temperature profiles are then presented for various individual kidneys in the sub-groups.

TABLE 1

KIDNEYS PROBED WITH 25 GAUGE THERMOCOUPLE FROM THE OUTSIDE AND A TEMPERATURE MAP MADE OF THE VARIOUS AREAS

Kidney #	WT. (gm.)	Cooling rate °C/min.	Lowest temp. (T_o)	Time stored t_{T_o} (hr.)	Final temp. (T_f)	Power map fig.(11)	$T_{max.}$ min.	Heating rate °C/min.	Temp. profile fig(8,9,10)	Glom	Tub.	Inter.	Vessel	Gen. Com.	Hist. plate fig.	Microfil plate fig.
8*	65	-	4	1/3	39.6	h8	52/36	142.4	p8a p8b	-	-	-	-	-	-	-
9*	75	-	4	1/3	34.6	h9	47/30	120	-	-	-	-	-	-	-	-
10*	43	-	4	1/3	33	h10	-	120	-	2	2	L	2	PV	7a	7b
11*	42	-	4	1/3	37	h11	-	155	-	3	2	L	2	V	-	-
16*	78	-	4	1/3	33	h13	-	144	-	2	2.5	L	2	PV	-	-
1	86	-	-79	15	25	h1	40/11	132	p1	2	2	?	?	PV	12	-
2	90	-	-79	15	33	h2	25/-18	102	p2	2.5	2.5	?	2	PV	13	-
3	32	1.38	-79	1	7.1	h3	19/-12	344	p3a p3b	2.5	2.5	?	2.5	PNV	-	-
4	36	1.0	-63	1	13.2	h4	36/4	269	p4a p4b	3	3	L	2.5	PNV	-	-
5	48	1.4	-95	2	23.9	h5	31/17	142	p5a p5b	-	-	-	-	-	-	-
6	81	1.34	-79	8	44	h6	52/31	231	p6a p6b	-	-	-	-	-	-	-
7	86	2.35	-40	12	5.1	h7	16/-14	104	p7a p7b	3	3	L	2.5	PNV	14a	14b
13**	87	1.5	-79	1	-5.6	h14	5/-14	125	p13a p13b	-	-	-	-	-	-	-
14**	92	1.5	-79	15	20	h15	36/-3	142	p14a p14b	-	-	-	-	-	-	-
15**	72	1.0	-79	1	16.0	h16	29/2	173	p14a p14b	-	-	-	-	-	-	-
17**	91	1.1	-79	24	19.0	h17	43/-8	139	p17a p17b	-	-	-	-	-	-	-
18**	91	1.88	-79	24	10.6	h18	36/-1	127	p18a p18b	-	-	-	-	-	-	-

* Kidney cooled to 4°C and perfused at this temperature for 20min. and then reward using microwave.

** Kidney sliced longitudinally in a medial-lateral plane after being thawed and temperature map made of the various areas.

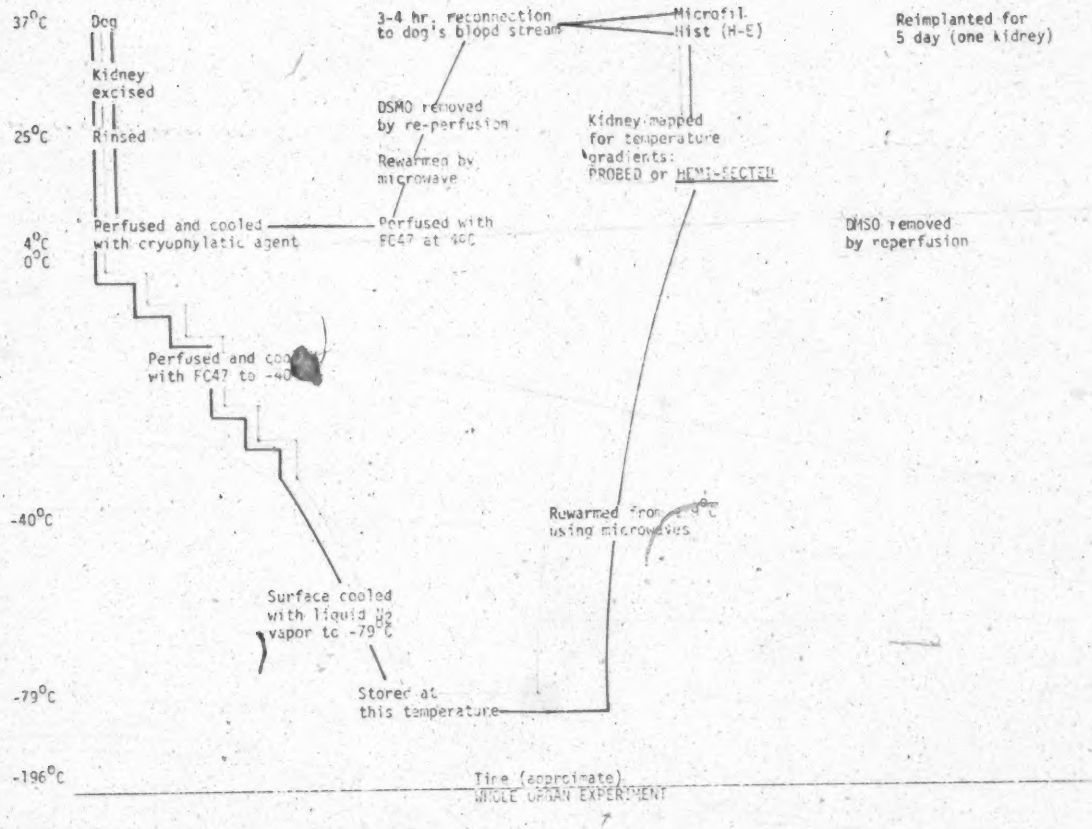


Figure 6. Kidney #8, 9, 10, 16——(1), Kidney #13, 14, 15, 17, 18——(2), Kidney #1, 2, 3, 4, 5, 6, 7——(3).

ANALYSIS OF DATA

A group of kidneys which were not frozen but the protective agent added, followed by perfusion at 4°C with FC47, and warming with microwave, showed viability after being anastamosed via the femoral artery and vein of the same dog for a number of hours. The histology

and microfil injection indicate a normal looking kidney (Figure 7a, 7b). The ultimate test of viability, however, is renal function after kidney reimplantation.

Heating rates of 100 - 200°C/minute have been obtained in thawing fluorocarbon loaded kidneys from -79°C. The kidneys which were not hemi-sected for the temperature profile, but probed from the outside to different depths, indicated no temperature gradients in the cortices, following thawing [Figure 8, 9(P3b to P7b)]. If the probe enetered the medulla, or pelvic region, cold spots were found [Figure 8, 9 (P1a to P7a)]. In an effort to locate these cold areas other kidneys were hemi-sected. When, in this new group of kidneys, perfusion of the protective agent was adequate, a uniformly thawed kidney resulted with a temperature gradient of ±10°C [Figure 10 (P13a, P14a, P17b)]. If there was inadequate perfusion during the initial rinse, and addition of the protective agent, cool spots resulted as can be seen in Figure 10 (P14b, P15a, P17a, P18a). Urine remaining in the pelvis also caused a large temperature gradient [Figure 10 (P13a)].

The histology and reimplantation of some kidneys for three hours indicated that the integrity of the kidney had been preserved (Figure 12, 13, 14a). When microfil was used to outline the micro-circulation (Figure 14b) it can be seen that the glomeruli and capillary plexus have been damaged with microfil leakage into Bowman's space.

Figure 7a. *Kidney #10 to Table #1.* Protocol as showen in Figure 6, line 1 (pink). Following rewarming with microwave, the kidney was reimplanted for three hours, removed and a microfil injection made of the micro-circulation. Histology - H&E was taken following this injection. *Interpretation* - glomeruli -2, tubules - 2, interstitium - normal, vessels - normal. General comment - microfil seen in the glomeruli, "viable".

Figure 7b. *Kidney #10 of Table #1.* Same kidney as in Figure 7a. Picture of microfil injection shows: (a) some well preserved glomeruli, (b) well preserved capillary plexus.

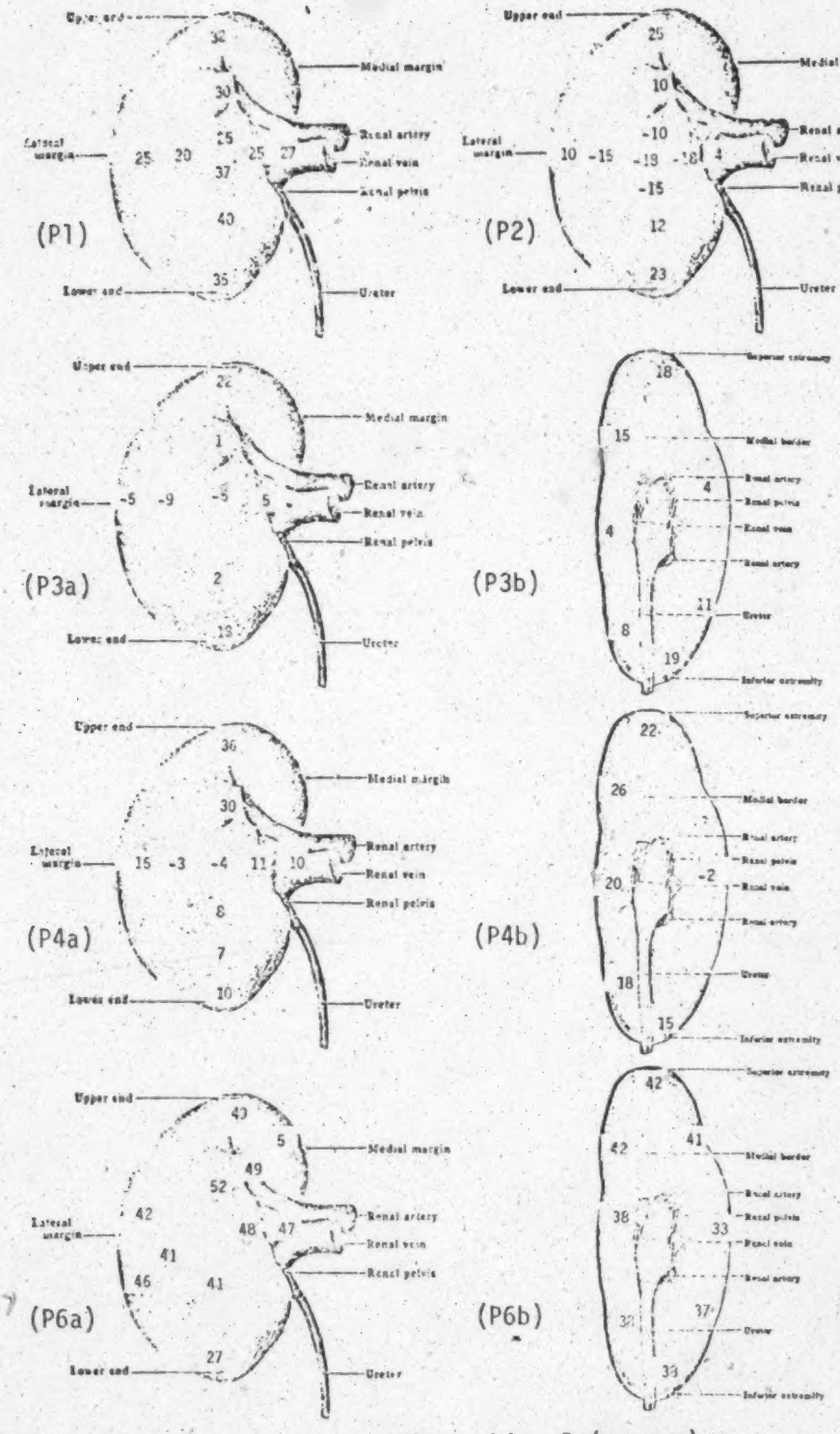


Figure 8. *Kidney temperature profile.* Line 3 (orange).

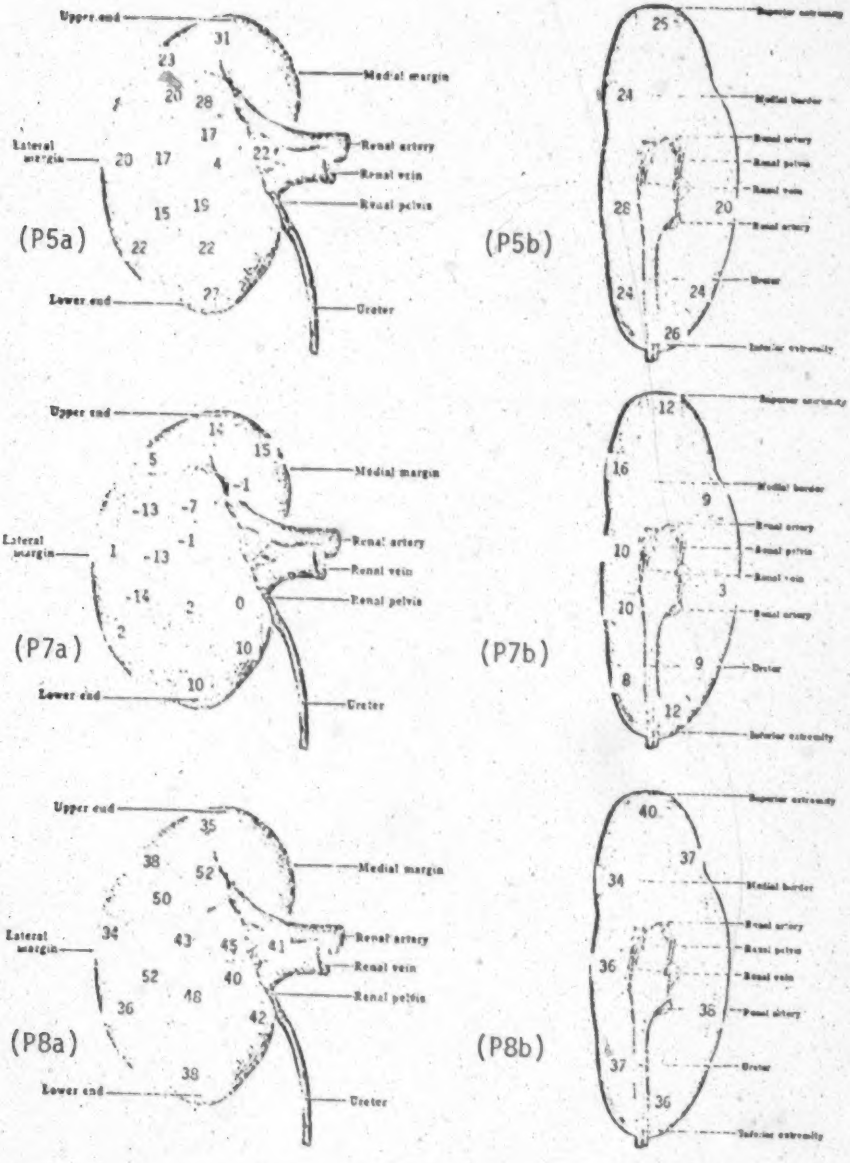
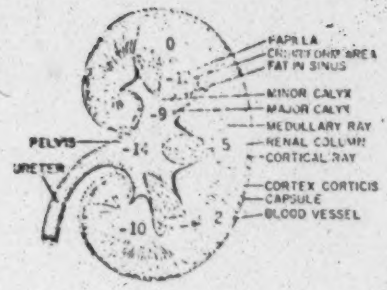
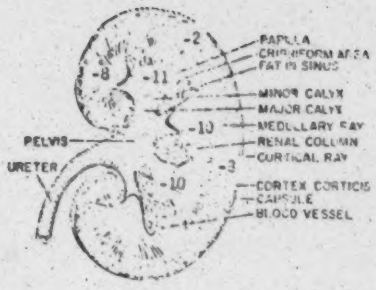


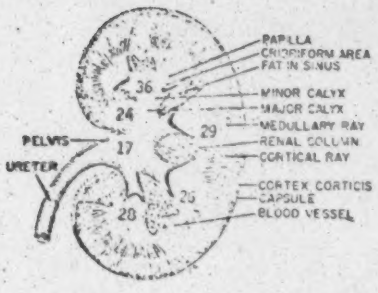
Figure 9. *Kidney temperature profile.* Line 3 (orange).



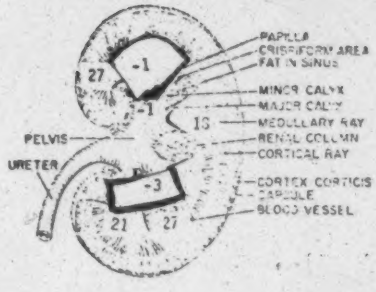
(P13a)



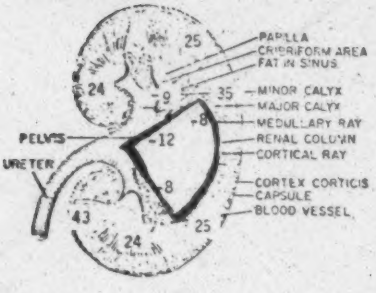
(P13b)



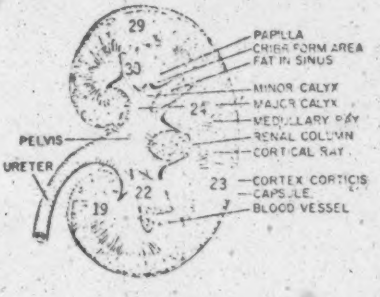
(P14a)



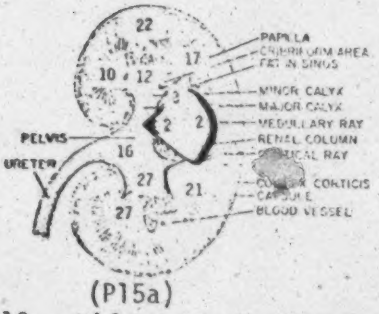
(P14b)



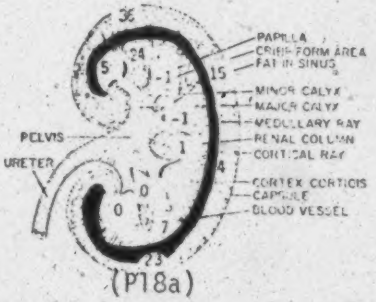
(P17a)



(P17b)



(P15a)



(P18a)

Figure 10. *Kidney temperature profile after hemi-section.* Line 2 (green).

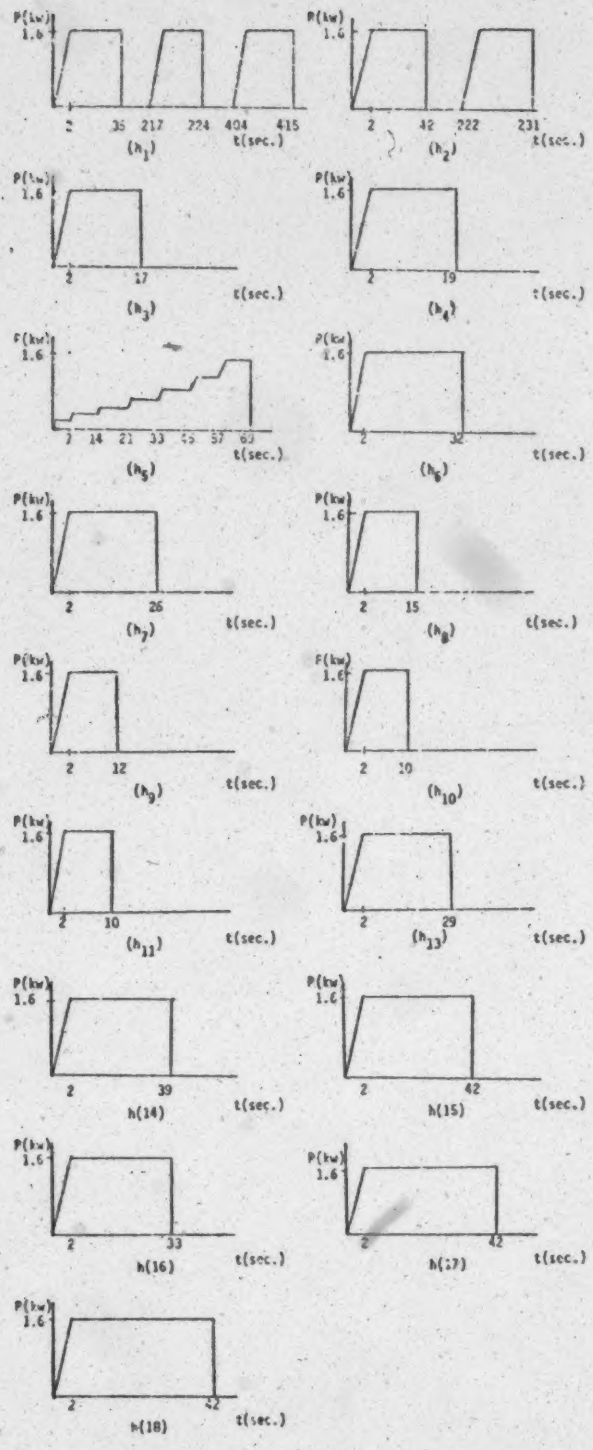


Figure 11. *Power input pattern.* (See Table 1).

Figure 12. *Kidney #1 of Table #1.* Handling of organs as shown in Figure 6, line 3 (orange). Kidney was reimplanted for several hours after thawing and temperature map was made, and then removed for H&E section. *Interpretation* - glomeruli - 2, tubules - 3, interstitium - loose, vessels - ?. General comment - as in Figure 1.

Figure 13. *Kidney #2 of Table #1.* Kidney handled as shown by Figure 6, Line 3 (orange). The kidney was reimplanted for three hours after thawing and probing with a 25 gauge thermocouple needle and then removed for H&E section. *Interpretation* - glomeruli - 3, tubules - 3, interstitium - loose, vessels - ?. . General comment - extensive glomeruli and intra-tubular hemmorhage indicating damage to the micro-circulation. "Probably not viable".

Figure 14a. *Kidney #7 of Table #1.* Kidney handled by protocol of Figure 6, Line 3 (orange). Following temperature probing with #25 gauge thermocouple needle, kidney was injected with microfil to outline micro-circulation. Histology H&E section was performed. *Interpretation* - glomeruli - 3, tubules - 2.5, interstitium - loose, vessels - ?. General comment - "Not viable".

Figure 14b. *Kidney #7 of Table #2.* Same kidney as in 14a. Picture of microfil injection. *Interpretation* - There is some well preserved glomeruli (a) with damage to other glomeruli, as can be seen with injection leakage into Bowman's space (b). Some portion of the capillary plexus can be seen (c) but not as much as the normal (Figure 17a, 17b, 17c).

Five day reimplanted kidney. Following the experimental procedure outlined below [Figure 15, Line 4, (orange)], a kidney was implanted into the groin of the same dog for five days. Upon removal, it was injected with microfil, followed by histology H&E section.

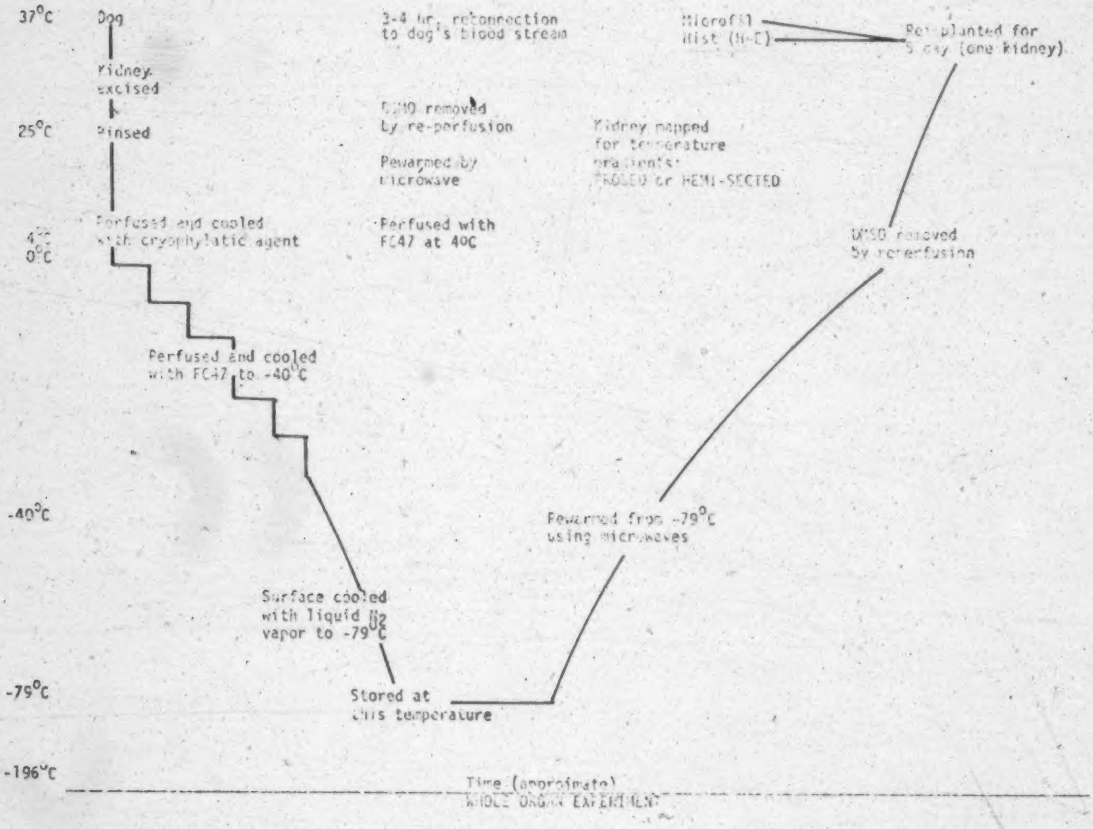


Figure 15. *Protocol of five day reimplanted kidney.* (4).

ANALYSIS OF DATA

A five day reimplanted frozen-thawed kidney shows damage to the micro-circulation (Figure 16). Some isolated areas look quite good with the glomeruli and capillary plexus well preserved, indicating that blood was being perfused through the kidney.

Figure 16. *Microfil injection of the five day implanted kidney.* There is still some vascular integrity in some areas with the glomeruli (a) and capillary plexus preserved (b) to some extent, but the capillary structure is not as normal as seen in Figure 17.

However, at no time was there evidence that the kidney was capable of forming urine, also in other parts of the kidney the microcirculation was grossly damaged and, in fact, could not be properly injected.

Figure 17a. *Normal unfrozen kidney (control).* Kidney injected to outline micro-circulation. 60 X mag. (a) - glomeruli, (b) - capillary plexus.

Figure 17b. *Normal unfrozen kidney (control).* Kidney injected with two colors of microfil. Red was injected into the artery and yellow via the vein. 40 X mag. (a) - glomeruli, (b) - capillary plexus.

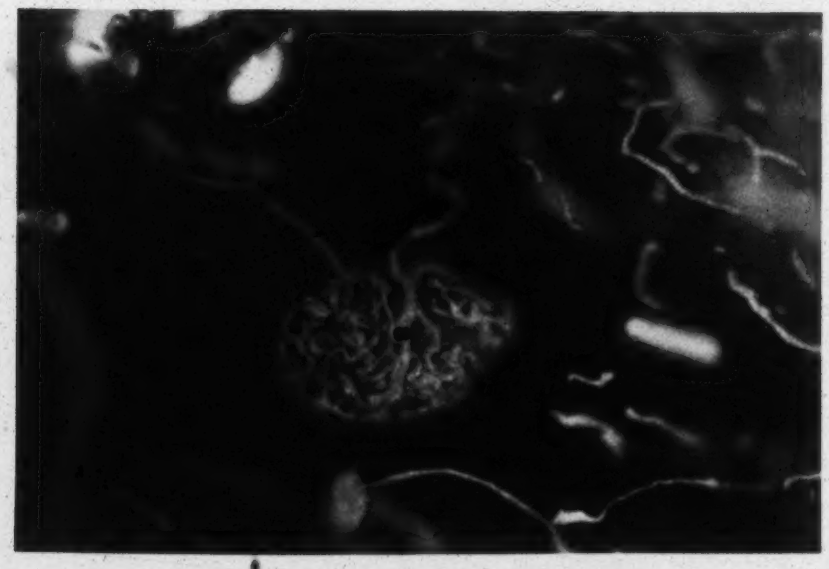


Figure 17c.. *Normal (control) kidney.* Microfil of one glomeruli - (a). 100 X mag.

Figure 17d. *Latex injection.* (a) - glomeruli, and (b) - capillary plexus of normal kidney.

Effect of DMSO on unfrozen kidney slices. An experiment on the effect of 10% and 15% DMSO in Cross solution on kidney slices when incubated for twenty minutes at room temperature, in an atmosphere of O_2. The Warburg apparatus was used and the O_2 and PAH# uptake were compared to normal slices incubated in Cross solution alone. The color code below (Figure 18) is used to indicate by which path the slices in Table #2 were processed.*

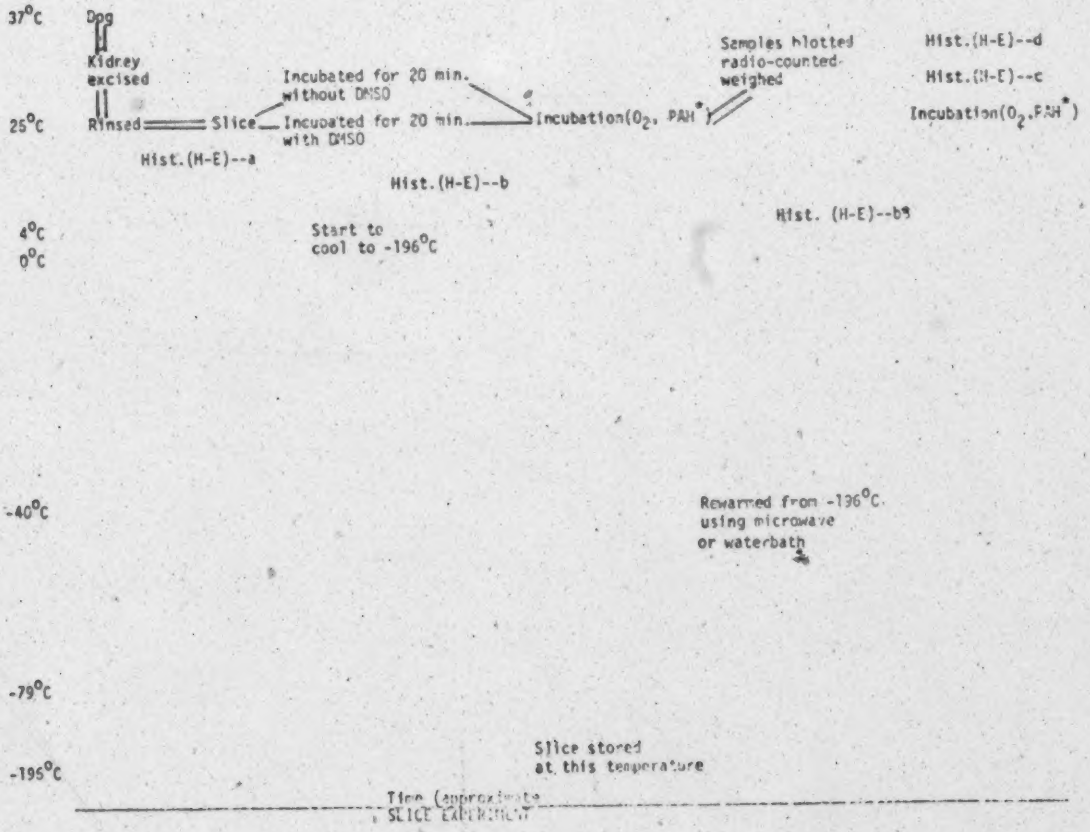


Figure 18. Kidney slice #75, 76, 77, 31, 32, 39, 40, 41 ——(5).
Kidney slice #36, 37, 38——(6).

* Appendix 3 Primary data for table 2

TABLE 2 EFFECT OF DMSO ON UNFROZEN SLICES

Sample #	Incubation soln. for 20 min.	O_2 at 30 min. cmm/mg	S/M	Glom.	Tub.	Inter.	Vessel	Gen. Com.
75	A	.75	7.76	3	3	L	2	NV
76	B	.86	9.63	3	3	1	2	V
77	B	.80	8.17	3	2	1	2	NV
31	B	1.47	12.5	-	-	-	-	-
32	B	1.0	15.1	-	-	-	-	-
39	B	1.48	9.2	-	-	-	-	-
40	B	.92	9.0	-	-	-	-	-
41	B	1.3	7.6	-	-	-	-	-
36	C	.63	7.3	-	-	-	-	-
37	C	1.4	5.37	-	-	-	-	-
38	C	.66	6.67	-	-	-	-	-

* All samples incubated in Warburg for 1 hr. in Cross soln.

Soln. A = 15% DMSO in Cross soln.

Soln. B = 10% DMSO in Cross soln

Soln. C = cross soln.

ANALYSIS OF DATA

Using O_2 and PAH# uptake in the Warburg apparatus as an index of viability, it would seem that incubation for twenty minutes at room temperature in 10% and 15% DMSO in Cross solution prior to the Warburg incubation has little effect on O_2 and PAH# uptake. In Table #2, the s/m is higher in most cases when the slices had DMSO added for the twenty minutes incubation. This seems to indicate that 10% and 15% DMSO is not toxic to the kidney slices.

In a few of the experiments in which histology studies were done, point (d) in flow graph (Figure 18), indicated poor histological preservation (Figure 19).

Figure 19. *Kidney slice #76 of Table #2.* The kidney slice processed as indicated by Figure 18, Line 5 (purple). *Interpretation* - glomeruli - 2, tubules - 3, interstitium - ?. vessels - ?. General comment - "Non-viable".

Rapid microwave warming of unfrozen kidney slices. Following Figure 20 below, a comparison was made between waterbath and microwave, in warming kidney slices from 4°C to room temperature. O_2 and PAH# uptake was used as the index of viability.

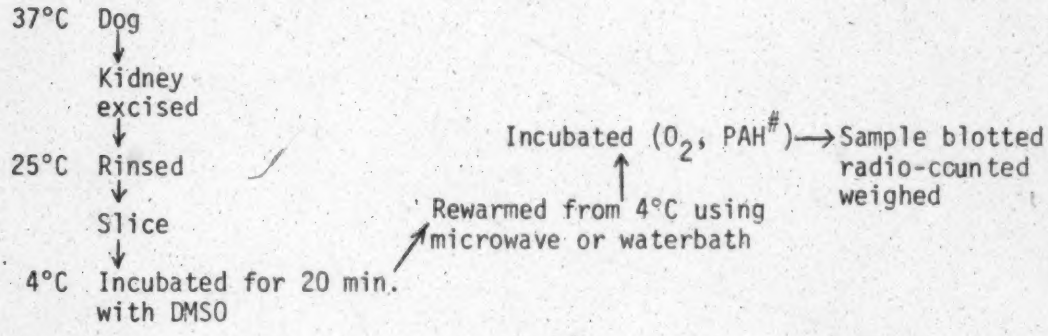


Figure 20. *Waterbath and microwave warming of kidney slices.*

TABLE 3 EFFECT OF MICROWAVE ON FROZEN SLICES WARMED FROM 4°C.

Sample #	O_2 mean (cmm/mg)	s/m mean
4*	1.59±0.57	-
4*	-	11.3±0.37
3"	1.56±1.0	-
4"	-	10.8±1.52

* - microwave warmed

" - waterbath warmed.

ANALYSIS OF DATA

From Table #3, a comparison was made between waterbath warming and microwave warming. There was practically no difference in O_2 and PAH# uptake when measured in the Warburg apparatus. This would seem to indicate that microwave is tolerated by this sensitive parameter of PAH# uptake.

Freezing and thawing of kidney slices. Experiments using frozen kidney slices checking the effect of freezing solution, concentration of protective agent, and fast or slow thawing using microwave or waterbath thawing. O_2 and PAH# uptake in the Warburg along with histological studies were used as an index of viability. All slices were processed as indicated in Figure 21, Line 7 (blue). The histology samples were taken as indicated in Figure 21, point (d).

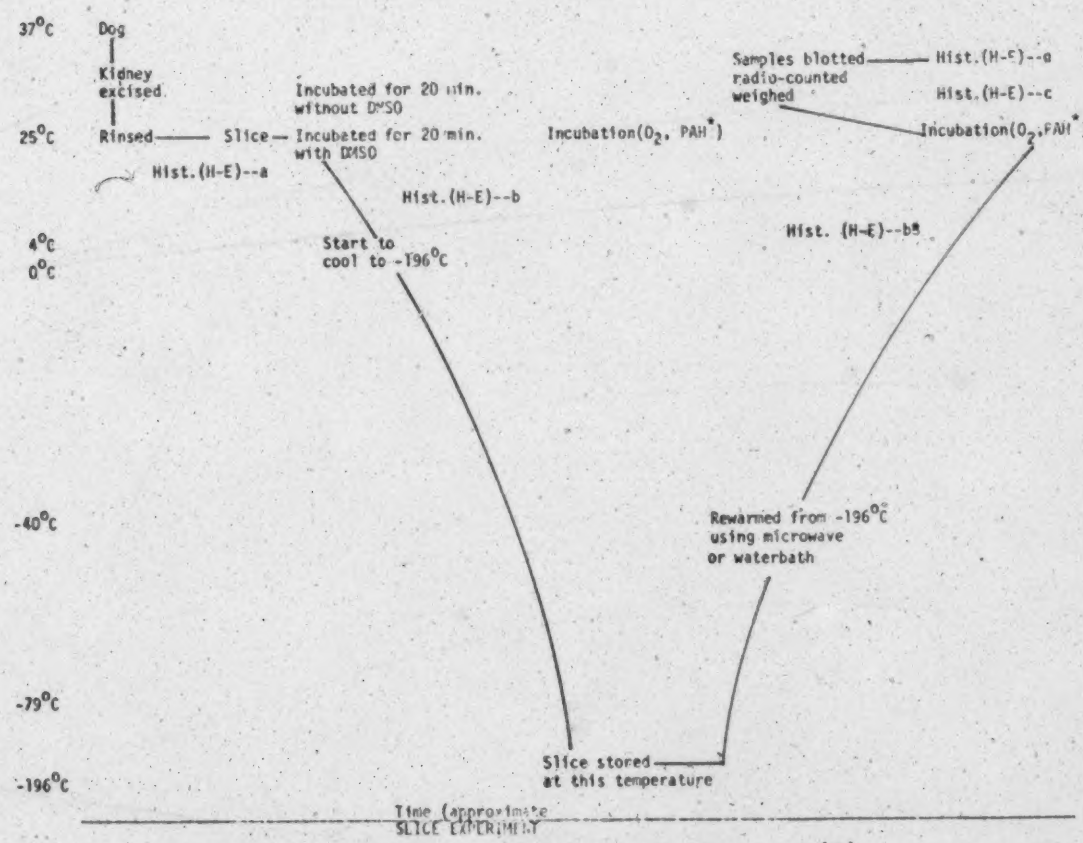


Figure #21. Kidney slices #81 - 102, Table 3. —— (7).

Table #4*indicates the solution each slice was incubated in for twenty minutes prior to freezing and also indicates the solution in which the samples were frozen. Cooling rates, final temperature and time stored at this temperature is also shown. The method of heating, heating rate, and final temperature after heating is indicated. The last columns illustrate O_2 uptake, s/m and an interpretation of the histology slides. These values can be compared to unfrozen slices of Table #2.

* Appendix 3 Primary data for table 4.

TABLE 4 RESULTS OF FROZEN-THAWED KIDNEY SLICES

Slice #	Incubation Soln. for 20 min.	Solution sample frozen in	Cooling rate oC/min.	T_o °C	Time stored t_{T_o}	Heating rate H_r °C/min.	Heating method WB or MW	Temp. after thawing T_f °C	O_2 at 30 min. cmm/mg	s/m	Glom.	Tub.	Inter.	Vessel	Gen. Com.	Hist. Plate fig.
81	A	A	.32	-79	1/4	331	MW(F)	4	.61	1.14	4	4	L	3	NV	-
82	A	A	.32	-79	1/4	414	MW(F)	4	1.06	1.57	4	4	L	3	NV	22
83	A	A	.32	-79	1/4	20.4	MW(S)	15	.51	1.2	4	4	L	3	NV	-
84	B	B	.32	-79	1/4	240	MW(F)	41	1.29	1.1	2	2	1	2	PV	23
85	B	B	.32	-79	1/4	317	MW(F)	32	.74	1.26	4	4	L	3	NV	24
86	B	B	.32	-79	1/4	21.1	MW(S)	16	1.4	1.55	3	3	1	2	NV	-
87	B	B	.32	-79	1/4	17.8	MW(S)	7	.80	1.25	4	4	L	3	NV	-
88	B	B	.37	-196	120	297	MW(F)	17	1.43	1.5	3	3	L	3	NV	-
89	B	B	.37	-196	120	202	MW(S)	17	.60	1.26	4	4	1	3	NV	-
90	B	B	.37	-196	120	63.6	MW(S)	-5	.50	1.56	3	3	L	2	NV	-
91	B	B	.37	-196	120	236.5	MW(F)	9	1.01	1.37	4	4	1	3	NV	-
92	B	B	.37	-196	120	221	MW(F)	14	.76	1.37	4	4	L	3	NV	-
93	B	B	.37	-196	120	220.3	MW(F)	50	1.19	1.31	3	3	1	2	NV	-
94	B	B	.37	-196	120	57.1	MW(S)	4	1.29	1.52	4	4	L	4	NV	-
95	D	D	.53	-196	120	151	MW(F)	4	.44	1.06	4	4	?	3	NV	25
96	D	D	.53	-196	120	151	MW(F)	4	.67	1.6	3	3	?	1	NV	-
97	D	D	.53	-196	120	44.4	MW(S)	4	.99	1.6	3	3	1	1	NV	-
98	D	D	.53	-196	120	110	WB(2min.)	25	.94	1.4	2.5	2.5	1	1	NV	-
99	D	D	.53	-196	120	110	WB(2min.)	25	.94	1.5	2	2	1	1	NV	-
100	D	D	.53	-196	120	156	MW(F)	51	.80	1.55	2.5	2.5	1	1	NV	-
101	D	D	.53	-196	120	140	MW(F)	27	2.1	1.6	2.5	2.5	1	1	NV	-
102	D	D	.53	-196	120	slow	slow	25	1.42	1.6	3	3	1	1	NV	-

Soln.A = 15% DMSO in Cross Soln.
Soln.B = 10% DMSO in Cross Soln.
Soln.C = Cross Soln.
Soln.D = MEM 10% DMSO, 10% fcs (Hepes)

WB(F) = Water bath warming fast
WB(S) = Water bath warming slow
MW(F) = Microwave warming fast
MW(S) = Microwave warming slow

ANALYSIS OF DATA

These kidney slices were frozen in different solutions and warmed by waterbath or microwave at different heating rates. O_2 uptake of the kidney slices in the Warburg, following thawing, was comparable to the unfrozen samples. PAH# uptake using s/m as the figure of viability was very poor, with little change noted with the type of freezing solution used or the method of thawing. Abbot, in 1969, reported a similar finding (52). The histology in this whole group of slices was very poor (Figure 22, 23, 24, 25), with the general comment of non-viability. The histology sample was taken at point (d) in Figure 21, as was the unfrozen slices of Table #2.

Figure 22. *Kidney slice #82 of Table #4.* The experimental procedure is as indicated in Figure 21, Line 7. *Interpretation* - glomeruli - 4, tubules - 4, interstitium - ?, vessels - ?. General comment - "definitely not viable".

Figure 23. *Kidney slice #84 of Table #4.* Experimental procedure as indicated by Line 7, Figure #21 and Table #3. *Interpretation* - glomeruli - 2.5, tubules - 3, interstitium - ?, vessels - ?. General comment - "Definitely not viable".

Figure 24. *Kidney slice #85 of Table #3.* Experimental procedure as indicated in Figure #21, Line 7, and Table #3. *Interpretation* - glomeruli - 2.5, tubules - 4, interstitium - loose, vessels - ?. General comment - "Definitely not viable".

Figure 25. *Kidney slice #95 of Table #4.* The experimental procedure is as shown in Figure #21, Line 7. *Interpretation* - glomeruli - 3, tubules - 4, interstitium - ?, vessels - ?. General comment - "Definitely not viable".

Histology deterioration. In Table #2 and #4, the sections were taken for histology at the end of the experimental procedure point (d), Figure #26. The following experiment was carried out to determine which step was responsible for the unexpectedly poor appearance of the tissue. The same protocol was followed as in Table #5* but the histology was taken at different points along the procedure. Figure #26 indicates what was done to each set of samples during the freezing thawing cycle, and where the histology was taken. Table #5 gives incubation solution for twenty minutes, the solution used to freeze the samples, cooling rates, lowest temperature and times stored at this temperature, heating rates, method of thawing and final temperature.

* Appendix 3 Primary data for table 5 .

The final lines give the histology interpretation at the different points along the flow graph. The letters a, b, ... etc., in Table #5, correspond to the same a, b, ... etc. in flow graph (Figure 26).

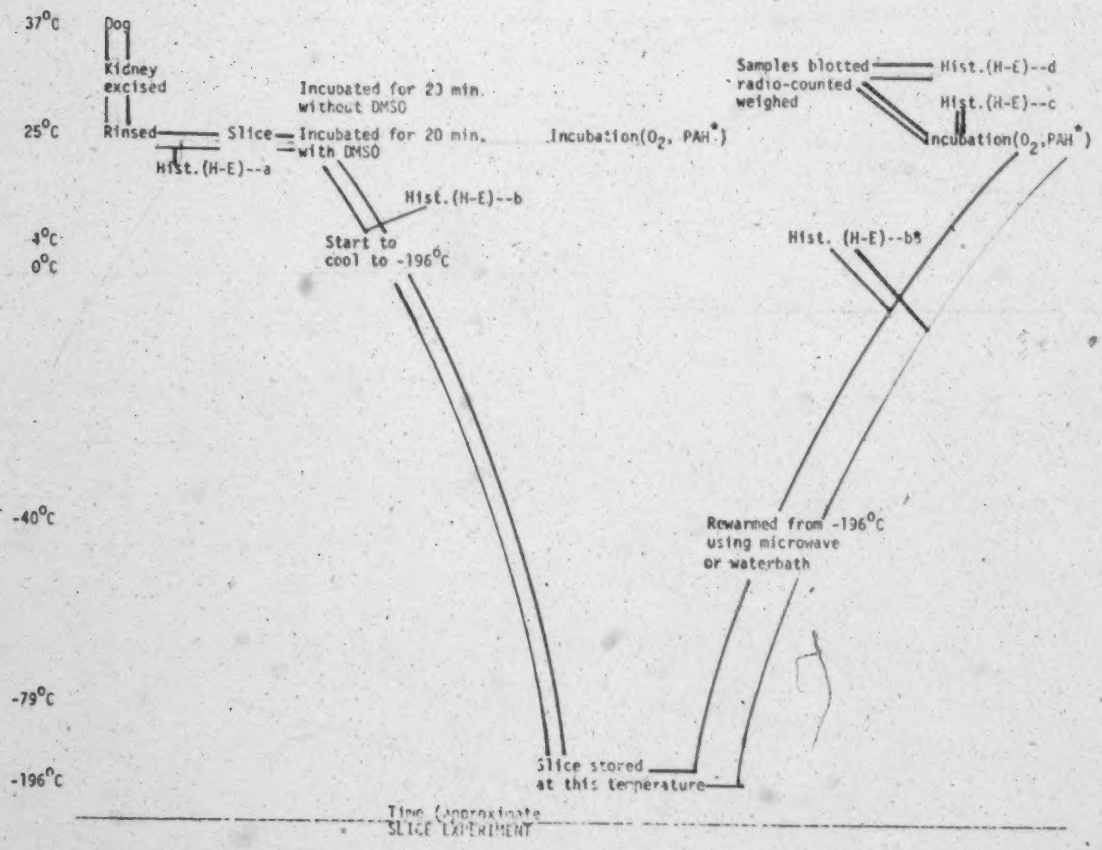


Figure 26. Kidney slices #102 - 113, Table 5.——(8). Kidney slices #114 - 117, Table 5. (9). Kidney slices #118 - 122, Table 5,——(10).

Table 5. Comparison of histology at different points along the experiem

Slice #	Incub. soln. 20 min.	Soln. frozen in	Cooling rate °C/min.	Time stored t_{st} (hr.)	Heating rate H_r °C/min.	Heating method WC or MW	Temp. after thawing °C	O_2 at 30 min. cpm/mg	s/m	Glomeruli a	b	b*	c	d	Tubules a	b	b*	c	d	Interstitium a	b	b*	c	d	Vessel a	b	b*	c	d	General Comment a	b	b*	c	d
102	A	D	.2	283	233	MW(F)	26	.71	1.4			2	3	1			1	2	1			-	L	-			1	?	1				MV	MV	V
103	B	C	.2	226	191	MW(F)	20	.77	1.2			1	4	4			1	3	3			-	L	*			1	?	?				MV	NV	MV
104	D	D	.2	280	231	MW(F)	15	.76	1.0			2	4	1			1	3	2			-	L	L			2	1	-				MV	NV	NV
105	B	D	.2	288	WB(2min.)	WB	25	1.01	1.02			2	4	1			1	3	2			L	L	?			1	?	2				PNV	NV	V
106	D	D	.2	208	WC(2min.)	WB	25	.91	1.18			2	4	3			1	3	3			L	L	L			1	?	3				PNV	NV	NV
107	D	D	.2	288	WB(2min.)	WB	25	1.13	1.01			2	3	4			1	2	3			L	L	P			1	1	?				MBV	NV	NV
108	E	E	.2	238	295	MW(F)	25	.83	1.05			2	3	3			2	3	3			?	L	P			1	?	?				V	NV	NV
109	E	E	.2	209	258	MW(F)	10	1.6	.93			1	3.5	3			1	3	2			?	L	P			1	?	?				V	NV	NV
110	E	E	.2	283	206	MW(F)	20	.47	1.0			1	3	3			1	3	3			1	L	1			1	2	2				V	PV	NV
111	E	E	.2	208	WB(2min.)	WB	25	1.1	.82			2	3	3			1	3	2			L	P	2			1	?	?				PV	NV	NV
112	E	E	.2	288	WB(2min.)	WB	25	1.15	.87			2	3	3			3	3	3			L	L	P			1	?	?				PV	NV	NV
113	E	E	.2	238	WB(2min.)	WB	25	2.1	.87			1	3	4			1	3	4			1	P	?			1	2	2				V	NV	NV
114	F	-	-	-	-	-	-	.93	6.1	1	1.5	-	2.5	3.5	1	1.5	-	2.5	3.5	L	L	-	L	L	1	1	-	2	2.5	V	V	-	PNV	DNV
115	F	-	-	-	-	-	-	leak	5.07	1	1.5	-	2.5	3	1	1.5	-	1.5	3.5	L	L	-	L	L	1	1	-	2	2.5	V	V	-	PNV	DNV
116*	F	-	-	-	-	-	-	1.5	3.7	1	2	-	2.5	3	1	2	-	2.5	3	1	L	-	L	L	1	1	-	2	3	V	V	-	PV	NV
117*	F	-	-	-	-	-	-	1.0	3.8	1	2	-	3	3.5	1	2	-	2	3.5	1	L	-	L	L	1	2	-	2	3	V	V	-	PNV	PNV
118	F	F	.5	216	201	MW(F)	4	1.46	1.25	1.5	2	2.5	2.5	3.5	1	2	2	2.5	2.5	L	L	L	L	L	1	2	2	2	3	V	PV	PV	NV	NV
119	F	F	.5	216	201	MW(F)	4	1.0	1.18	1.5	2	2.5	2.5	3.5	1.5	2	2.5	3	3.5	L	L	L	L	L	1	2	2	2	2	V	PV	PV	PNV	NV
120*	F	F	.5	216	172	MW(F)	4	.53	1.07	1	2	2.5	3	3.5	1.5	2	2.5	3	3.	L	L	L	L	L	1	2	2	3	3	V	PV	PNV	NV	NV
121*	F	F	.5	216	155.8	MW(F)	4	.76	1.1	2	1.5	2	3.5	4	1.5	2	2.5	2.5	3	L	L	L	L	L	2	1	2	3	3	V	V	PV	NV	NV
122*	F	F	.5	216	166.7	MW(F)	17	1.7	1.08	2.5	1.5	2	3	3.5	2	2	2.5	3	3	L	L	L	L	L	1	1	2	2	2	PV	PV	PV	PNV	NV

* Samples incubated in Warberg in Media 199 + 10% fcs
Soln.D =MEM + 10% fcs + 10% DMSO
Soln.E =MEM + 10% fcs +10% DMSO + 1% pluronic + 20% FC 47
Soln.F =Med. 199 + 15% DMSO + 10% fcs
& Not frozen
foot note: All samples frozen to -197°C.

ANALYSIS OF DATA

All the kidney slices in Table #5 took up O_2 following thawing, but again the slices that were frozen (brown and black) did not take up PAH# in the Warburg. The histology was quite different in this set of experiments. The histology was much better preserved if taken right after thawing, point (d), Figure 27a, 27b, 27c, 28b, 28c.

The samples which were not frozen (yellow, Figure 26), produced a similar result. The histology prior to the Warburg was much better than just after incubation.

From the above data, it would seem that care must be taken in using histology for assessing damage incurred by the freeze-thaw insult. The damage in actual fact may not be due to freezing or thawing.

This data establishes that histologic appearances deteriorate markedly with incubation. Although sections stained after thawing but before incubation looked as if they were "probably viable", there is no way in which the microcirculation can be assessed in slices. Histology after thawing can be falsely interpreted as normal; histology after incubation can be falsely interpreted as too abnormal; histology is clearly a very difficult parameter to assess in this aspect of research. The uptake of PAH# is still the most sensitive index of tubular cell viability and these experiments failed to show preservation of viability by this parameter (see discussion).

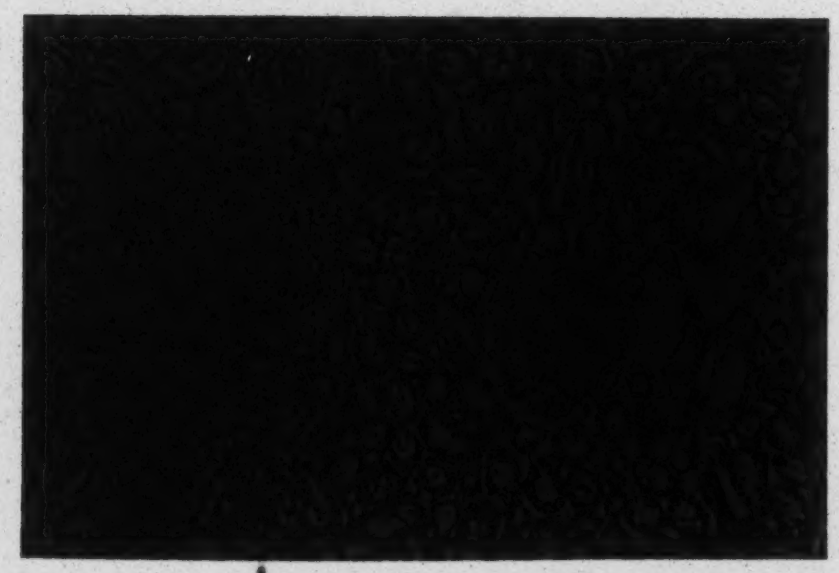


Figure 27a. *Kidney slice #104, Table #5.* The experimental protocol was as shown in Figure 26, Line 8 (brown). Histology was taken at point b*. *Interpretation* - glomeruli - 1, tubules - 2.5, interstitium - loose, vessels - ?. General comment - "Possibly viable."

Figure 27b. *Kidney slice #104, Table #5.* Same slice as in Figure 27a, except the histology taken at point c in flow diagram (Figure 26). *Interpretation* - glomeruli - 3.5, tubules - 3.5, interstitium - loose, vessels - ?. General comment - "Definitely not viable."

Figure 27c. *Kidney slice #104, Table #5.* Same kidney as in Figure 27a, except histology taken at point (d) in flow diagram. *Interpretation* - glomeruli - 3.5, tubules - 4, interstitium - loose, vessels - 3. General comment - "Definitely not viable."

Figure 28a. *Kidney slice #107, Table #5.* The experimental procedure was as indicated by Figure 26, Line 8 (brown). The histology was taken at point b*. *Interpretation* - glomeruli - 1.5, tubules - 2.5, interstitium - loose, vessels - 1. General comment - "Possible viable."

Figure 28b. *Kidney slice #107, Table #5.* Same slice as in Figure 28a, but histology taken at point c, in flow diagram, Figure 26. *Interpretation* - glomeruli - 3, tubules - 3.5, interstitium - loose, vessels - ?. General comment - "Definitely not viable."

Figure 28c. *Kidney slice #107, Table #5.* Same slice as in Figure 28a. Histology taken at point (d) in flow diagram, Figure 26. *Interpretation* - glomeruli - 3, tubules - 3.5, interstitium - loose, vessels - ?. General comment - "Definitely not viable."

CHAPTER IV

MICROWAVE THAWING OF TISSUE CULTURE CELLS

It is reasonable to postulate that the use of frequencies in the range 500 to 3000 MHz offers the greatest promise for the eventual defreezing of organs from storage banks at low temperatures, if such preservation and storage is at all possible. Fundamental to these attempts at organ preservation is the effect of the microwave insult, when the power coupled to the organ is of the order of 10 watts/gm in the presence of high electric field strengths in the resonant cavity of a microwave oven. To investigate the microwave insult, it was decided to compare the viability of tissue culture cells thawed in a microwave oven from -79°C to temperatures between 0 - 37°C at comparable rates to those obtainable in a waterbath at 35°C (the orthodox method of thawing for most cell systems).

METHODS

(a) Tissue Cultures. The technique of cultivating animal cells on a solid surface using a fluid overlay was originated and developed by Earle and co-worders (61 to 65). The method is adaptable to the growth of a wide spectrum of established cell lines and of primary dispersed tissue cells. It has the advantage of being simple and reproducible and at the same time permits

detailed microscopic study of cells when they are grown on a cover glass.

In the monolayer technique cells attach to the glass, or other surfaces, flatten and grow to yield a continuous sheet, or network, usually one cell thick. Cells in such a monolayer may be spindle shaped, polygonal or amoeboid. When crowding occurs the cell layer may loosen from the surface and slough off.

Cells are harvested from the monolayer by scraping or by treatment with an enzyme solution such as trypsin, pancreatin or collagenase. The choice of harvesting will depend, in some measures on the cell strain being used and the composition of the medium.

For this experiment Chinese hamster cells (Puck strain A) were used to assess the relative viability of microwave thawing in comparison with a 35°C waterbath.

Cells were grown in monolayers until a sufficient number of cells in the logarithmic growth phase could be harvested by trypsinization (0.1% Difco :250). Cells were grown in Eagle's minimum essential medium (Biocult Laboratories) supplemented with 10% fetal calf serum (Biocult of Microbiological Assoc. Inc.) and buffered with 0.01 M tricine, organic buffer (Sigma Chemical Co.). Cells were plated at a concentration of between 200 and 500 cells per dish (Falcon plastic flasks 30 ml). Macroscopic colonies were stained with methylene blue after 8 - 9 days of culture at 36°C, and the colony formation scored.

(b) Freezing regime. Cell suspensions (20,000 cells in 2 ml) in ampoules with either DMSO (5% and 10%) or DMSO (5%) and hydroxyethyl starch (5%) or an extracellular cryophylactic agent, polyvinyl-pyrrolidone (PVP), were frozen in solid CO_2 with alcohol for at least 30 minutes. Seeding was not used. The cooling rate was approximately 20°C per minute as defined by the time taken for the sample to reach -79°C from 23°C when the temperature was measured, in a duplicate, with a copper-constantin thermocouple.

(c) Microwave thawing apparatus. Microwave thawing was achieved at rates from 100 to 300°C/minute in a modified 1 kw microwave oven operating at 2.45×10^9 Hz (or cycles per second). The 2 ml frozen samples, in vitro, were placed in an insulating styrofoam container (transparent to the microwave field) and rotated at 15 rpm by a teflon rod connected to an external electric motor. Certain procedures were taken to protect the microwave generator, as the sample absorbed only about 1% of the available power: in the majority of the tests a 50 ml sample of water at 20°C was placed in the oven. A thermal sensor was used to protect the microwave generator, which is capable of withstanding very high reflected power for short periods.

The microwave heating system is a low power and simplified version of the one used the thaw the canine kidneys uniformly at comparable rates when DMSO is one of the cryoprotective agents.

The transfer of a sample from the ice bath to the heater was less than 10 seconds. After heating, the temperature of the

TABLE 6

MICROWAVE THAWING OF CHINESE HAMSTER CELLS FROM -79°C : SURVIVAL

Test #		Freezing rate	Heating method	Additive	Heating rate °C/min	Final Temp °C	Survival: mean # of colonies	± Standard error
1	a	Unfrozen	Control		--	--	301	7.6
	b	20°C/min	MW	10% DMSO	190	0	281	24.9
	c	"	"		216	9	245	8.6
	d	"	"		265	29	217	33.0
	e	"	"		265	30	150	4.0
	f	"	"		280	36	128	20.6
	g	"	"		290	40	37	3.7
2	a	Unfrozen	Control	10% PVP	--	--	342	11.9
	b	"	"	10% DMSO	--	--	448	3.4
	c	20°C/min	WB	"	115	35	388	19.9
	d	"	MW	"	240	13	433	6.0
	e	"	MW	"	250	25	414	11.6
3	a	20°C/min	WB	5% DMSO	115	35	287	34.7
	b	"	MW	"	218	34	286	7.0
	c	"	MW	"	113	11	333	8.0
	d	"	MW	"	193	34	305	34.8
	e	"	WB	5% DMSO&HES	115	35	374	35.8
	f	"	MW	"	163	35	295	17.7
	g	"	MW	"	205	27	362	40.1
	h	20°C/min (Control Frozen)	WB	none	115	35	45	8.6
	i	"	MW	"	250	25	5	1.6

In all instances between 3 and 5 plates per ampoule were assayed.

All cells frozen in tissue culture media and minimal Eagles plus 10% fetal serum.

(1) WB = Normal thawing in water bath at 35°C

(2) MW = Microwave thawing

2 ml sample was recorded within 5 seconds by a sterilized thermocouple. About 50% of the samples were heated to a useful final temperature, between 0 and 40°C. Others were rejected, if the measured final temperatures were higher than 40°C.

(d) Details of the colony scoring, or counting assay. The method of scoring was determined by colony formation. Following thawing of the 2 ml sample (20,000 cells), a dilution was made to give 200 to 500 cells in each incubation flask. The cells were then cultured for 8 - 9 days, followed by staining with methylene blue. The colonies formed on the glass were visually counted, making the assumption that each colony came from one surviving cell. The number of colonies formed were compared to the same sample which had not been frozen, cultured and then stained.

ANALYSIS OF DATA

With 10% DMSO (Tests 1 & 2) the survival after microwave heating ranged from 70 - 95%. Above 0°C, it is to be noted that the microwave survival tended to decrease as the final measured temperature increased. It may, therefore, be advantagious to stop the rapid thawing process as soon as possible after 0°C. Test 3 shows that, even with microwave thawing, 5% DMSO is satisfactory and the some additional survival occurs when 5% Hydroxethyl Starch is present.

The results presented show that there are no disadvantages to microwave thawing with this cell system. Presumably, the

absorption of energy resulted in rapid and uniform thawing. Survival figures as good as, or better than, those obtained with standard waterbath thawing procedures for Chinese hamster cells were obtained. More than twice the conventional thawing rate was successful with microwaves. It is to be noted that, theoretically, this is relatively independent of sample, shape, and size. Further, microwave heating has the advantage over short wave diathermy, one of the other physical systems that has been used (45), in that contacting electrodes are not required; the handling procedure is thus quick and simple.

There is no reason to believe that results would not be similar if cultured monolayers of renal epithelial cells were used instead of Chinese hamster cells. However, such experiments have not been done. But it must be borne in mind that though microwave re-warming may well turn out to be equally applicable to larger tissue masses (such as organised heart or kidney tissue) there is evidence that DMSO, so evidently successful with cell suspensions, has recently been shown to be less efficacious in one solid organ system, the rabbit kidney [Pegg (44)], as the intima of the vascular endothelium of small vessels is apparently better preserved by glycerol than by DMSO. Such experiments show the hazards of extrapolating results from one system to another.

DISCUSSION

In the search to establish possible methods for preservation of mammalian organs in a deep frozen state, and their subsequent recovery, it has been concluded that electromagnetic energy in a limited frequency range is essential for both the uniformity of thawing and the control of the thawing rate. From a consideration of the penetration depth of the wave, frequencies in the range from 500 to 5000 MHz (5 to 50 X 10^8 cycles per second) are of interest. In this thesis a convenient frequency in the middle of this range, 2450 MHz in the ISM band, has been chosen. With the above system it has been possible to thaw kidney from -179°C to above zero at a heating rate of 100 - 200°C/minute, while still maintaining uniformity to within ±10°C.

In the process of testing out the uniformity of thawing, it became apparent that a uniformly thawed kidney only resulted when pre-freezing perfusion had been uniform and complete.

When the kidney was cut in half, following thawing, it was clearly seen that areas which still had traces of blood, indicating incomplete perfusion, were those that remained frozen. Kidneys which had good perfusion during the initial rinse, during addition of the protective agent and during the cooling with FC47, were those which thawed uniformly with a ΔT of less than ±10°C.

The difficulty in assaying the damage due to the freeze-thaw insult, previously pointed out by Abbot (52), is clearly verified in these experiments. Prolonged reimplantation following this insult was tried on only one canine kidney. The result

indicated that some vascular integrity was maintained, even though the functional capacity of the kidney did not return. Clearly, much more research is needed before kidneys can be made to survive the freezing insult but thawing by microwaves, after adequate perfusion, may be accepted as a partial solution to one aspect of this highly complex problem.

The kidney slice experiments were designed to study another aspect of the overall problem, namely protection of renal epithelial cells from freeze-thaw insult. In these experiments, it was decided to assess viability by three parameters: oxygen uptake and uptake of radio-labelled para-aminohippurate, after slice incubation in a Warburg apparatus; and the microscopic state of the tissue by hematoxylin and eosin staining of suitable fixed tissue sections.

In control experiments, unfrozen slices were shown to tolerate 10% and 15% DMSO as indicated by the O_2 and $PAH^{\#}$ uptake. With the frozen-thawed slices, using different freezing and thawing rates, and different protective agents, O_2 uptake was comparable to normal, but there was no $PAH^{\#}$ uptake. The failure to preserve this tissue function was a real disappointment; it remains unsolved. Experiments showed that microwaves, when used to warm from 4°C up to 30°C, did not affect the $PAH^{\#}$ uptake capability, and there is no reason to believe that microwaves, per se, would specifically affect this function when used on frozen tissue. Some support for this hypothesis that microwaves would not cause intrinsic damage to frozen tissue, per se, was also obtained when tissue culture

cells were used as a method of assay. Comparison between microwave and waterbath thawing suggested that the two had similar effects, with 70 - 95% recovery in microwave thawing. Further work to overcome the damage to the PAH# uptake mechanism is urgently indicated. If this barrier cannot be overcome, then further research with whole kidneys would seem to be pointless.

A system that may prove very useful at a level intermediate between cell suspensions and adult whole organs is the use of fetal mouse hearts implanted in the ears of syngeneic mice. Freezing and thawing rates, protective agents, and the effect of the microwave insult, could well be studied with return of electrical activity of the hearts used as the criteria of survival. The problem with the above system is that the electrical activity is a physical phenomenon only indirectly related to the pumping function of a heart. The system, however, does consist of an organised multicellular structure, but one which lives without the need for vascular anastomosis as it is nourished by diffusion and by the ingrowth of new capillaries of the host origin. This system is one which may be explored if the problem of preserving the kidney slice capacity for PAH# uptake cannot be overcome.

A useful by-product of research on kidney slices was the definition of the value of routine light microscopic sections, stained by hematoxylin and eosin. It was found that Warburg incubation, even when PAH# uptake was good, caused marked deterioration in the appearance of the tissue when stained after

incubation. But it was also found that the relatively well preserved histologic appearance of freeze-thawed tissue (which was stained immediately after thawing) was also misleading as even tissue, well preserved according to this parameter, did not take up PAH# when subsequently incubated. Reliance cannot be placed on routine light microscopic appearance as indicating preserved viability, though tissue that appears obviously damaged will certainly prove to be non-viable by the more sensitive parameters that must also be employed.

CONCLUSION

A uniformly frozen canine kidney with controlled freezing rates can be produced with the systems used in these experiments. Uniformly and rapidly thawed kidneys were only obtained when initial perfusion had been complete and uniform, but function did not occur in one subjected to prolonged reimplantation. Unfrozen kidney slices tolerated 10 - 15% DMSO and were not damaged by microwave warming from 4°C up. PAH# uptake never returned to normal following the freeze-thaw insult, and this remains an unsurmounted barrier to further research with kidney slices. At the cellular level, using DMSO and HES as protective agents, and with freezing rates of 20°C/minute, microwave thawing from -79°C was comparable to thawing with a waterbath. Thus, there is no reason to suppose that the failure of kidney slice preservation was necessarily due to the use of microwaves.

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(Power describes his successful experiments of 1661 on freezing and then thawing eel-worms, Turbatrix Aceti. This is the first known scientific record of the subject, and predates the work of Robert Boyle and Antony van Leeuwenhoek.)

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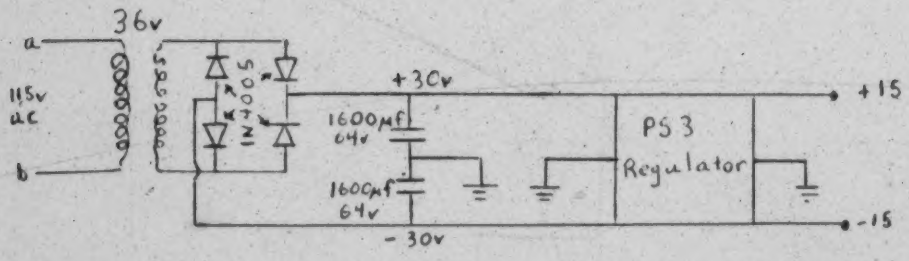
APPENDIX 1

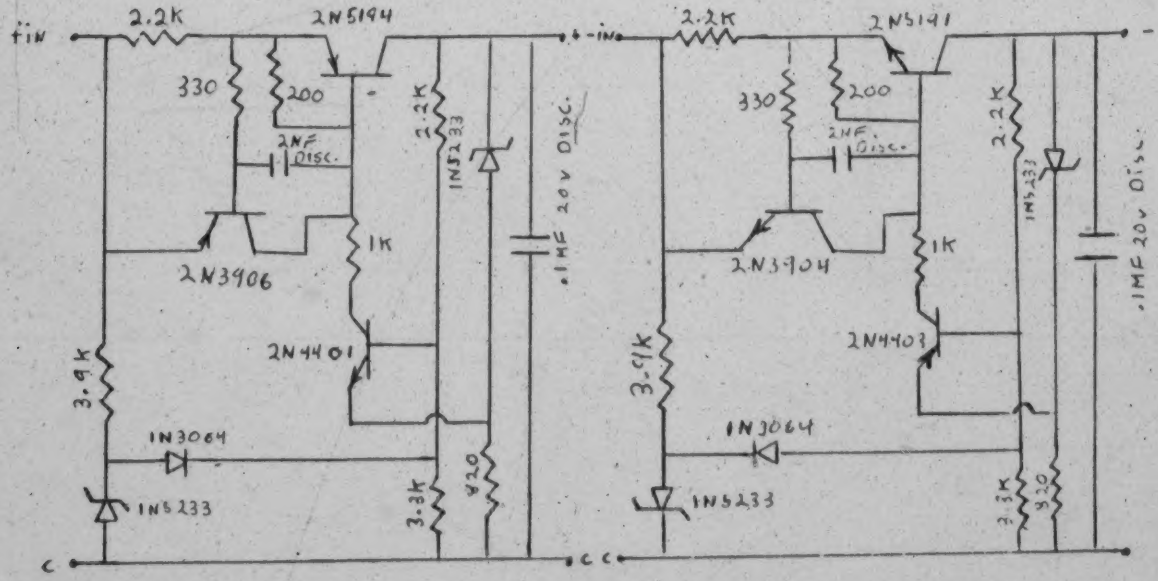
THERMOCOUPLE DIFFERENCE AMPLIFIER

Power Supply



Current limiting to 250 ma, ±15v power supply, short circuit protected.

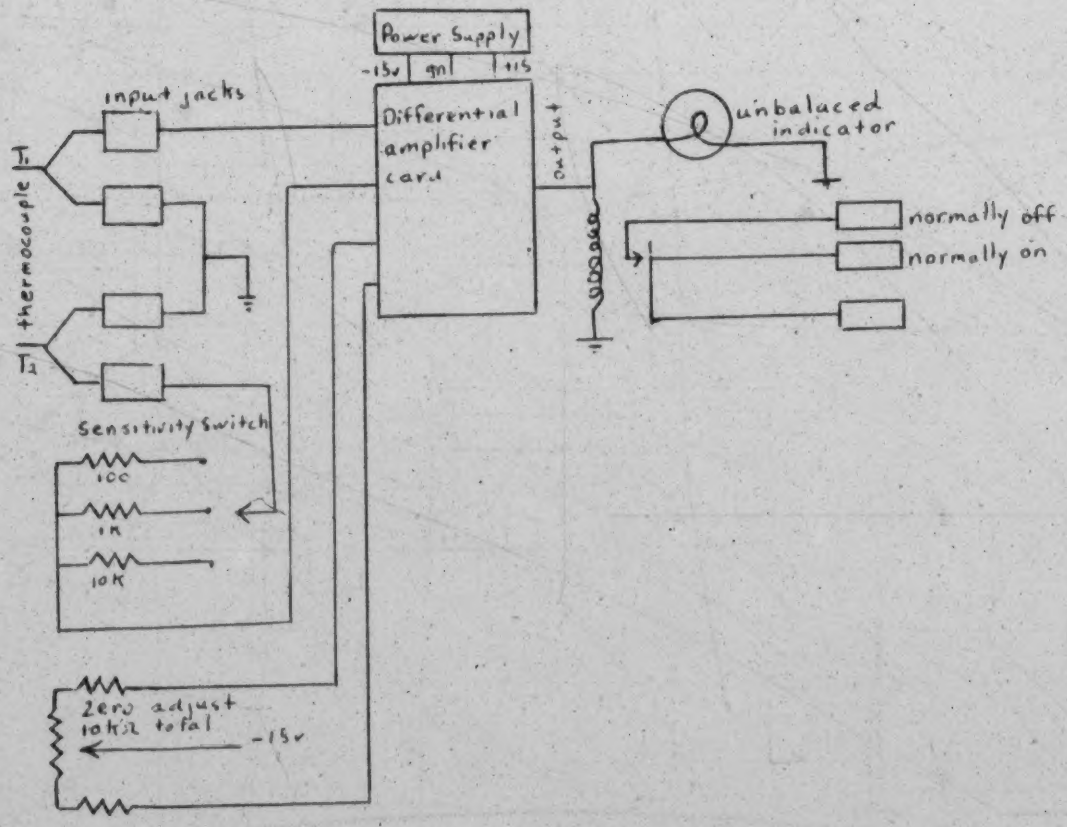
PS3 Regulator



Positive Regulator

Negative Regulator

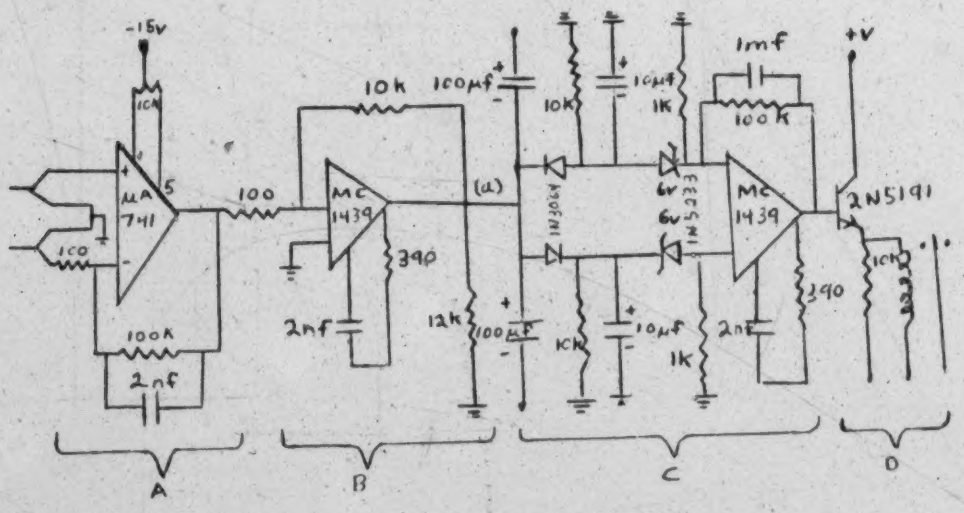
Block diagram of system.



The sensitivity of the differential amplifier can be adjusted so that any desired temperature difference between T_1 and T_2 can be set. The output of the differential amplifier runs a relay which then runs the liquid njtrogen solinoid valve.

T_1 and T_2 cupper constant needle thermocouples.

Circuit for difference amplifier card.



A - high voltage gain (1000) differential amplifier, gain adjustable.

B - high gain amplifier.

C - circuit to insure that output is always positive. If there is a positive voltage at (a) the signal passes through the lower diode and if the voltage is greater than 6 volts the signal passes through the zenor diode and through the operational amplifier non-inverting terminal getting a positive signal at the output to run the relay. If there is a negative voltage at (a), the signal passes through the upper diode and through the inverting terminal of the operational amplifier and one receives a positive signal at the output to run the relay. The above circuit always presents a positive signal to the relay.

D - transistor that is used as a relay driver which turns the solenoid valve on and off.

APPENDIX 2

MICROWAVE HEATING SYSTEM DESIGN

Two resonant microwave systems have been used for thawing and warming biological samples. Both were cubic structures; the basic design following that of the conventional microwave oven. The smaller unit, a ten inch cube, coupled to a 1 kw, 2450 MHz magnetron, was used for thawing tissue culture cells. The larger volume oven (80 liters) was used in organ thawing. This seventeen inch cube was coupled to a 2 kw magnetron at 2450 MHz.

Both of the above systems were equipped with rotating turntables on which the samples were placed. In the larger cavity, the turntable was made to oscillate as well as rotate. This was accomplished by having a shaft powered by two independent electric motors (Figure 4). Rotation speeds were in the range of 10 - 20 rpm and the oscillation rate was selected between 15 and 30 cycles per minute.

The turntable and drive shafts were made of teflon which is a material that has a low dielectric constant. When 2 ml in-vitro samples were heated (tissue culture cells) a thermal insulating styrofoam cover was used. This material also has a very low dielectric constant. These small samples may effect the field patterns and the number of modes present in the cavity. Heating times were determined experimentally in advance, and the samples that were not recovered in the range 0 to 40°C were rejected. With this system heating rates between 50 and 500°C/minute were obtained.

When dealing with larger samples such as the kidney, the seventeen inch cubic cavity was used. The kidneys were placed in a nylon container which was then fitted into a one liter beaker of fluorocarbon which was at 4°C. This compound is much like teflon and absorbs very little power.

The ten inch and seventeen inch cubic structures were chosen for the following reasons: uniformity of heating and high energy conversion appear to be best achieved in specific size cubic cavities. The interaction between the field patterns of different modes tends to minimize the variation of the electric and magnetic fields, and thus increases the uniformity of heating; that is, the more modes that can be excited, the more even the heating will be.

The resonant frequency of an empty cavity is given by the dispersion relation: $(\omega/c)^2 = (\pi\ell/a)^2 + (\pi m/b)^2 + (\pi n/d)^2$ (1) where $\omega = 2\pi f$, a, b, d are the dimensions of the cavity; ℓ, m, n are the integers corresponding to TE(ℓmn) and TM(ℓmn) modes. Equation 1 has been analysed for microwave ovens (57) where it has been shown that if a certain set of integers for ℓ, m, n are chosen, the three dimensions a, b, d of the cavity, maximise the number of degeneracies which can be obtained. The total number of modes N when plotted against frequency f have step discontinuities as seen in Figure 29.

The steps are due to mode degeneracies giving rise to different resonant field patterns at the same frequency, i.e. specific integer sets (ℓ m n) which solve Equation 1.

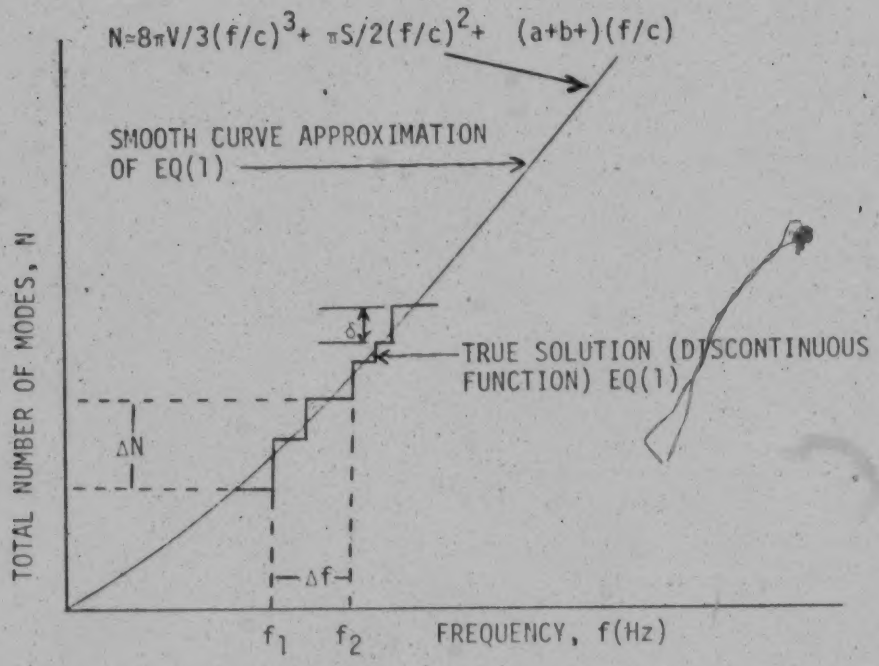


Figure 29. *An illustration of Equation 1.* Bolt (66) and Maa (67).

The step height δ in Figure 29, is an even integer 0, 2, 4 in the microwave case. By solving equation 1 it has been found that the maximum value of δ corresponds to 18 modes for all cavities having volumes less than or equal to 100 liters excited at 2.45 GHz, i.e. since there are two types of waves TE and TM, 18 modes are obtained by doubling the distinct permutations of each bracketal set (ℓ, m, n). If the calculations are extended to a bandwidth of 100 MHz (2.4 to 2.5 GHz), it has been shown (57) that the dimensions for an 80 liter cavity can give 68 modes within this bandwidth.

Solving Equation 1 for different integers, it can be shown that the first appreciable mode density at 2450 MHz occurs when P = 17 and 18 for a cubic cavity. If a = b = d, Equation 1 reduces to $\ell^2 + m^2 = n^2 = 4(a/\lambda)^2 = P$, where P is a positive integer. At P = 17, (ℓ m n) = (104) (223) and P = 18 (ℓ, m, n) = (114) (330). For

a few values calculated for P the following table 6 results.

P	(ℓmn)		P	(ℓmn)		P	(ℓmn)		
1			24	(224)		47			
2	(101)		25	(340)		48	(444)		
3	(111)		26	(105)	(134)[a]	49	(236)		
4			27	(115)	(333)[a]	50	(345)[b]	(505)[b]	(107)[a]
5	(102)		28			51	(117)	(515)[a]	
6	(112)		29	(234)	(250)[a]	52	(460)		
7			30	(125)		53	(146)	(270)[a]	
8	(220)		31			54	(127)	(336)	(525)[a]
9	(122)		32	(440)		55			
10	(103)		33	(225)	(414)[a]	56	(246)		
11	(113)		34	(334)	(350)[a]	57	(227)	(544)[a]	
12	(222)		35	(135)		58	(370)		
13	(230)		36	(244)		59	(137)	(355)[a]	
14	(123)		37	(106)		60			
15			38	(116)	(235)[a]	61	(346)[b]	(560)	
16			39			62	(156)	(237)[a]	
17	(104)	(223)[a]	40	(260)		63			
18	(114)	(330)[a]	41	(126)	(443)	64			
19	(133)			(450)[a]		65	(108)	(256)	(470)[a]
20	(240)		42	(145)		66	(118)	(147)[a]	
21	(124)	(430)[a]	43	(335)		67	(337)		
22	(332)		44	(226)		68	(280)		
23			45	(245)	(360)[a]	69	(128)	(247)[a]	
			46	(136)		70			

a = third type, b = second type

Table 6. *Mode integers of a cubic cavity.* (57)

In practice the magnetron has a bandwidth of about 15 MHz so that an appreciable number of modes are coupled by the radiator. If P is set equal to 17 at 2450 MHz, d is equal to 9.937 inches and at P = 18, d becomes 10.225 inches. If the average is taken for these two P values, a cubic cavity of 10.08 inches would give the maximum mode density. In actual practice the cavity was made ten cubic inches to allow for expansion of the walls during heating. This was the reason for using a ten inch cubic cavity in the tissue culture work, as this size of cavity gave the maximum number of

modes at his frequency.

The second large mode density occured when $49 \leq P \leq 54$, Table 6. The largest mode density occurs at P = 50, and to get maximum number of modes at 2450 MHz, a cubic cavity of 17,042 inches was used. Maintaining this size of cavity, the frequency needed to satisfy Equation 1 when P = 51 is 2475 MHz and at P =49 the frequency must be 2425 MHz. If the magnetron has a bandwidth of 15 MHz, a large mode density will result with a 17.042 inch cubic cavity.

As in the small cavity, the cavity was made seventeen inches to allow for wall expansion during heating. In the kidney work, a seventeen cubic inch cavity was used to try and get the maximum mode desnity, with the hope of getting a uniformly thawed kidney. In the large seventeen inch cavity a folded strip line antenna was used to obtain good coupling to the maximum number of modes. The size of the actual design is given in Figure 30.

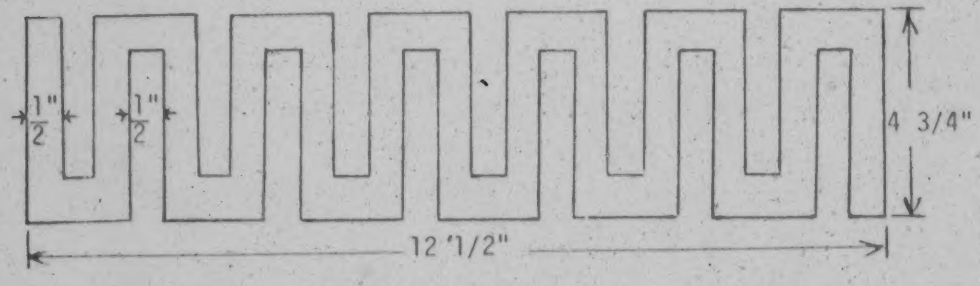


Figure 30. *Dimensions of the slow wave radiator used in the seventeen inch cavity.* The distance of the radiator from the cavity wall was one-half inch. The thickness of the aluminum slow wave radiator was 0.065 inches.

With the above slow wave radiator good energy conversion

was obtained with little effect noted on the reflected power as to the type of load used, and if that load was stationary or moving.

The above considerations apply to an empty or lightly loaded cavity. Generally a cavity that couples to the magnetron when empty will couple to a wide range of loads, but uniformity of heating in this work was only obtained if the load was moved through the cavity volume. In the case of thawing kidneys uniformly, it was necessary to use two planes of rotation to obtain an energy conversion of 60 - 70%.

In thawing tissue culture cells, very high field strengths were applied to see the effect on survival. The ten inch cube cavity achieved this by rotating the 2ml sample in the oven, with a 50ml water load in one corner of the cavity. This gave rise to preferential heating of these samples. Heating rates of around 200°C/minute were obtained which was comparable to waterbath warming.

Assuming that the kidney is mostly water, the assumption was made that 80gm of tissue are almost equal to 80cc of tissue. Therefore, in the 80 liter cavity this gave 0.1% filling factor. If a plot of energy conversion efficiency η versus percent filling factor under stationary conditions of the load, is made, it can be seen that at 0.1% filling factor there should be 50% energy conversion (Figure 31). With the rotating and oscillating turntable a 60 to 70% energy conversion was obtained. The kidney absorbed 60 to 70% of the power which was = 600 watts. Figure 31 indicates that the higher the filling factor the higher the energy conversion, however, it has been shown that a filling factor need not be greater than 3% to achieve 98% energy conversion.

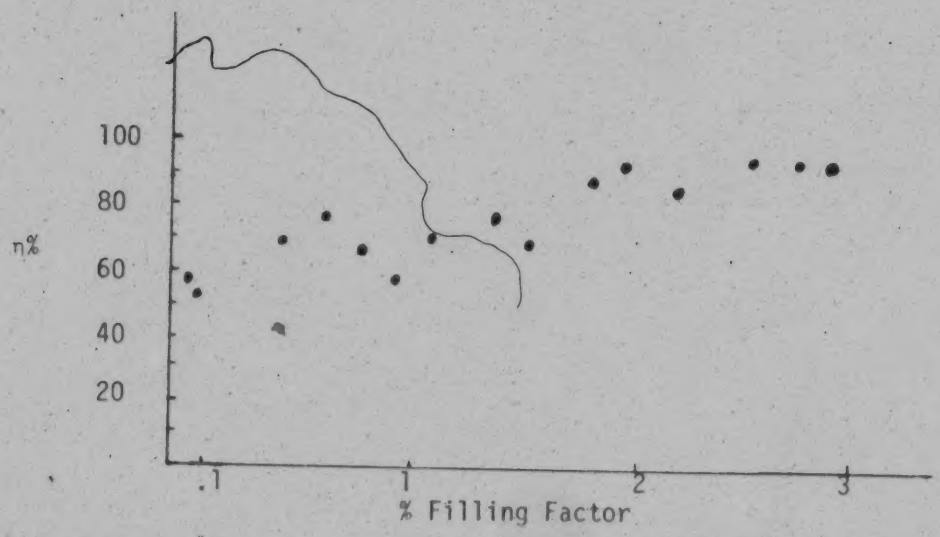


Figure 31. *Plot of cavity energy conversion efficiency using water samples initially at 20°C.* (57)

A directional coupler was used to measure the incident and reflected power (Figure 32).

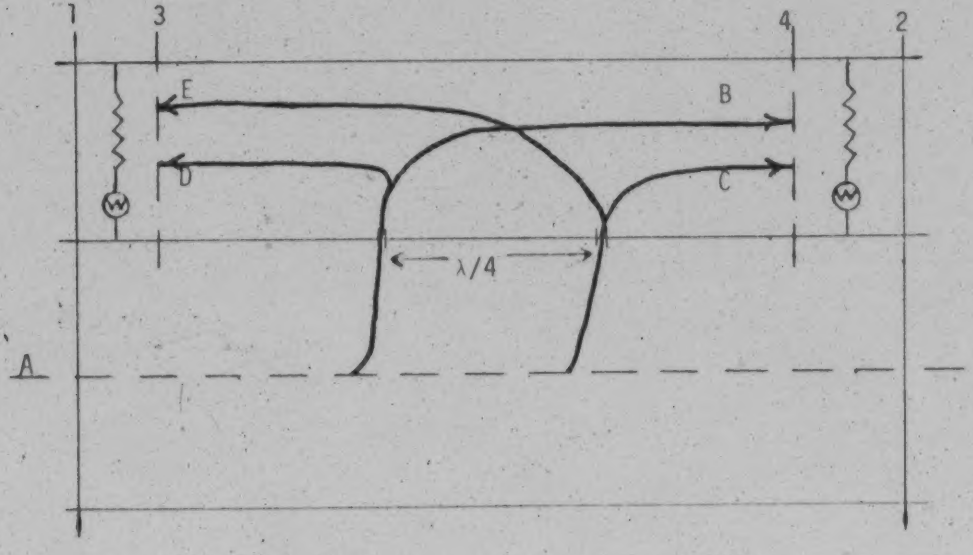


Figure 32. *Directional coupler.*

A directional coupler is used to separate the forward and backward travelling waves in a guide. There is a main waveguide with two small holes placed a quarter wave apart coupling to an auxillary guide terminated at each end by a matching resistance and meter as shown. If wave A progresses toward the right coupled waves from the two holed at terminal 4 follow path B and C of equal lengths, and the contributions add in that load, its meter recording the strength of A. The coupled waves through the two holes interfer at terminal 3, however, since the paths E and D differ in length by a half wave length, and the coupling through the two holes are substantially the same in amounts, if the holes are small. By symmetry, a wave flowing to the left will register at terminal 3 but yield coupled waves which cancel at 4. Therefore meter 4 reads the strength of the wave to thr right, and meter 3 that to the left. This system was used to give some indication of incident and reflected power.

APPENDIX 3

Table 2

Sample #	Counts per vial of incubation solution.	Counts per tissue sample.	Counts per mg of tissue.	Counts per mg of incubation solution.	s/m
75	29564	62526	230	29.6	230/29.6
76	29259	67108	281	29	281/29
77	37177	46425	302	37	302/37
31	71460	111901	876	72	876/72
32	84850	86409	1266	85	1266/85
39	60599	84782	557	61	557/61
40	61374	114808	549	61	549/61
41	62269	74119	473	62	473/62
36	52645	122649	382	53	382/53
37	69798	56869	371	70	371/70
38	52785	76253	347	53	347/53

Table 4

Sample #	Counts per vial of incubation solution.	Counts per tissue sample.	Counts per mg of tissue.	Counts per mg of incubation solution.	s/m
81	44559	18215	51	45	51/45
82	46537	13185	73	47	73/47
83	33448	15669	40	33	40/33
84	44112	8719	49	44	49/44
85	43575	16462	55	43	55/43
86	47543	11714	74	48	74/48
87	19457	6373	24	19	24/19
88	182559	54143	272	183	272/183
89	168890	74590	212	169	212/169
90	167876	49100	262	168	262/168
91	155738	49728	213	156	213/156
92	149466	71827	204	149	204/149
93	146783	50729	258	197	258/197
94	204254	49550	310	204	310/204
95	37377	5916	39	37	39/37
96	35097	7472	57	35	57/35
97	34113	6351	58	34	58/34
98	32655	10881	46	33	46/33
99	31664	10939	47	32	47/32
100	30273	13048	47	30	47/30
101	36230	5082	56	36	56/36
102	33999	6904	52	34	52/34

Table 5.

Sample #	Counts per vial of incubation solution.	Counts per tissue sample.	Counts per mg of tissue.	Counts per mg of incubation solution.	s/m
102	107140	31200	150	107.14	150/107
103	100800	17631	121	100.8	121/100
104	100000	25100	100	100	100/100
105	115600	24190	118	115.6	118/115
106	107600	30607	127	107	127/107
107	111800	21357	113	111	113/111
108	120950	33528	127	121	127/121
109	117200	19838	109	117	109/117
110	118000	30090	118	118	118/118
111	201200	27225	165	201	165/201
112	117200	11424	102	117	104/117
113	119500	11616	104	119	104/119
114	8496	4240	52	8.5	52/8.5
115	402	262	2.3	.4	2.3/.4
116	1007	417	3.7	1.0	3.7/1.0
117	991	397	3.8	1.0	3.8/1.0
118	79643	14176	99.8	79	99.8/79
119	75346	17829	90	75	90/75
120	84313	14623	90	84	90/84
121	77888	15849	89	78	89/78
122	80807	9917	87	81	87/81